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Multimodal Image Analysis of Chronic Leukemic Lymphoproliferative Disorders and the Hypothesis of »Single« and »Multiple« Programmed Stops in the Development of Typical and Atypical Forms of Leukemias and Lymphomas

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A B S T R A C T

The study consisted of morphometric analysis, assessment of the argyrophilic nucleolar organization region (AgNOR) characteristics, and image cytometry (ICM) in different tumor mass compartments: bone marrow (BM), peripheral blood (PB) and lymph nodes (LN) from patients with chronic leukemic lymphoproliferative disorders. A total of 71895 cells were analyzed on SFORM PC (VAMSTEC, Zagreb). Correlation between morphometric, AgNOR and ICM characteristics revealed the cells with low proliferative activity to possess small, homogeneous AgNOR, with the majority of cells in the peak of DNA histogram. The cells with high proliferative activity had inhomogeneous AgNOR, mostly containing greater DNA content than peak cells, pathologic mitoses (DNA>4N), or the majority of cells were in the S-phase of the cell cycle. Cells with medium proliferative activity and annular AgNOR were in-between. Analysis of different tumor mass compartments showed that lymphatic cells with the affinity to accumulate in BM regularly exhibited low proliferative activity (a lower percentage of cells in SFC and highest percentage of cells in the peak of the G_0/G_1 phase). The cells in LN exhibited the characteristics of proliferative cells (an increased number of AgNOR, larger and more proliferative inhomogeneous AgNOR, and lowest percentage of cells in the G_0/G_1 phase). The migration of cells from BM to LN and between lymph nodes occurred through PB (there were cells with low and high proliferative activity: a higher proportion of cells in SFC and at the same time in the G_0/G_1 phase of the cell cycle). Analysis of cell size and proliferative activity in different compartments of tumor mass revealed that the cells in BM and PB did not differ substantially according to size and proliferative activity, while an inverse pattern was observed between PB and LN. As small cells are inactive and larger cells more proliferative, the analysis quite unexpectedly showed the PB cells to be largest and most inactive, in contrast to LN where the cells were smallest and most active. The »single« and »multiple programmed stops« have been hypothesized in the development of typical forms of leukemias and lymphomas and atypical forms of subacute and subchronic leukemias. Differentiation impairment may occur at any stage, and different »stop« locations result in different morphology and affinity to accumulation in bone marrow, peripheral blood and lymph nodes.

Key words: *multimodal image analysis, chronic leukemic lymphoproliferative disorders, single and multiple programmed stops, subacute and subchronic leukemias*

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Introduction

Historically, disorders were termed leukemias when their clinical presentation included a typically abnormal and frequently increased number of white blood cells in the peripheral blood, contrary to lymphomas in which cases enlargement of lymphatic organs was present as a predominant abnormality[1]. The World Health Organization (WHO) classification of hematopoietic neoplasias describes chronic lymphocytic leukemia (CLL) as leukemic neoplasm composed of monomorphic small, round to slightly irregular B lymphocytes in the peripheral blood (PB), bone marrow (BM), spleen and lymph nodes (LN), admixed with prolymphocytes and paraimmunoblasts forming proliferation centers in tissue infiltrates[2]. In the WHO classification, CLL is always a disease of neoplastic B cells, whereas the entity formerly described as T-CLL is now called T-cell prolymphocytic leukemia. Other chronic leukemic lymphoproliferative diseases (CLLPD) that can masquerade as CLL are hairy cell leukemia, or leukemic manifestations of mantle cell lymphoma, marginal zone lymphoma, splenic marginal zone lymphoma with circulating villous lymphocytes, or follicular lymphoma. To achieve an accurate diagnosis and prognosis[3-5], it is essential to evaluate the blood count, blood smear, and the immune phenotype of the circulating lymphoid cells[6,7]. Morphologically, CLL can be classified into typical and atypical forms. Typical CLL (CLL-TYP) is the most common morphological form of CLL with less than 10% of prolymphocytes of peripheral blood lymphatic cells. Atypical CLL (CLL-ATYP) comprises two subtypes: chronic lymphocytic leukemia/prolymphocytic leukemia (CLL/PL) with increased prolymphocytes in peripheral blood (10–55%) and atypical CLL with lymphoplasmacytoid differentiation and/or presence of cleaved cells (CLL/LP)[8].

Morphometric analysis allows for the objective quantification of a set of parameters, or features representing the cell. The analysis showed that each class of cells is associated with a set of unique parameters, which provide significant information in the analysis of lymphoproliferative disorders and may serve as an additional tool for the morphological evaluation of diseases[9]. Chronic CLL-TYP and ATYP as well as other CLLPD are low grade neoplasms with a diploid DNA index and low but variable proliferative activity[10]. Apparently, most cells are in G_0 phase, but recent studies comparing expression of cell cycle regulatory proteins in these diseases with that of normal resting and stimulated lymphocytes suggest that the neoplastic cells are arrested in early G_1 phase or at the G_0/G_1 transition[10–12]. The argyrophilic nucleolar organization regions (AgNOR) are widely studied as a proliferation marker to analyze the proportion of cells in proliferative cell cycle, in tumor pathology in general, as well as in lymphoproliferative disorders[10,13,14]. Conventional kinetic studies are irrelevant in B-CLL since the great majority of circulating cells are in G_0 or G_1 phases, which are indistinguishable from one another by flow cytometry determination of DNA content[12]. The new parameters of modified analysis of diploid histogram seem to be appropriate for kinetic analysis by the method of image DNA cytometry, and with newly characterized types of AgNOR points, homogeneous, inhomogeneous and annular, play a role in calculating the proliferative-kinetic index of lymphoproliferative disorders[15].

The aim of the study was to identify the specific morphometric, proliferative and/or kinetic parameters in different tumor mass compartments, i.e. bone marrow, peripheral blood and lymph node. Then, the proliferative and kinetic parameters were used in an attempt to assess the origin and distribution of malignant lymphatic cells in various clinical and morphological entities.

Material and Methods

The study was performed on cytologic smears of bone marrow, peripheral blood and lymph node FNA samples collected from patients with leukemic forms of CLLPD classified according to WHO classification. The analysis was carried out in the entire CLLPD population, and in the B-CLL-TYP and B-CLL-ATYP subgroups separately. The following parameters obtained by computer analysis were assessed: image morphometric characteristics of the cell and nucleus (IM); image characteristics of AgNOR (IAgNOR); and image DNA cytometry (ICM). A total of 657 smears (71895 cells) were analyzed by the three methods in different tumor mass compartments (bone

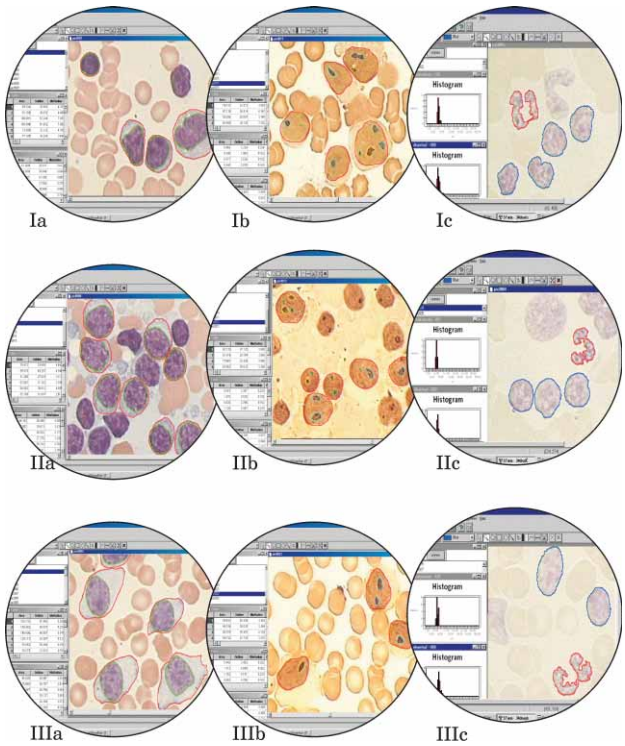

Fig. 1. Image analysis of patients with B chronic lymphocytic leukemia/prolymphocytic leukemia: I) bone marrow, II) lymph node, III) peripheral blood, a) morphometry, b) AgNOR, c) image DNA cytometry).

marrow, lymph node and peripheral blood) (Figure 1). Averages of 100 cells were analyzed for IM and IAgNOR, and 120–250 cells for ICM. Fresh and archival smears stained according to May-Grünwald-Giemsa (MGG) were used for morphometric analysis, according to Feulgen (Merck, Germany, Kit N.1.07907/3) for ICM, and silver impregnation for IAgNOR[16]. For morphometric analysis, the cytoplasmatic and nuclear margins were interactively marked (the cytoplasm first, then the nucleus), with manual correction. The following parameters of nuclei as well as of cytoplasms of cells were analyzed: area, outline, convexity, minimal and maximal radius, length (L) and breadth (B), regularity factors (FF-4π area/r^2 and elongation FF-L/B), and nucleus to cytoplasm ratio (N/C)[17]. The number, area (minimal, maximal and mean), and AgNOR to nucleus ratio were analyzed for each AgNOR type (homogeneous, inhomogeneous and annular); then total number of all AgNORs; total area (minimal, maximal and mean); and ratio of total area of all AgNOR and nuclear area. A modified method of diploid type DNA histogram analysis according to Kardum-Skelin[15] was employed, with determination of DNA index (DI), synthetic (S) phase according to Cornelisse et al.[18], and cell percentage of DNA greater than 4N. Granulocytes (n≥20) were used as internal control. The analysis was done on a PC using the SFORM supporting software (Vamstec, Zagreb, Croatia). Statistical processing of the results was performed by the Statistica 7.1 program (StatSoft, Inc., Tulsa, USA).

Results

Correlation of the IM, IAgNOR and ICM image parameters analyzed

(a) In total cell population of leukemic forms of chronic lymphoproliferative diseases (CLLPD)

Spearman rank correlation was used among morphometric characteristics, AgNOR characteristics and tumor cell DNA histogram in various tumor mass compartments, i.e. bone marrow, peripheral blood and lymph nodes (Figure 1), in CLLPD patients and in separately in the subgroups with B-CLL-TYP and B-CLL-ATYP. The highest rate of statistically significant correlations was recorded in peripheral blood lymphatic cells (Table 1). Morphometric characteristics of the cell and nucleus showed negative correlation with homogeneous AgNOR characteristics (larger cells and nuclei had a lower number and area of homogeneous AgNOR), whereas the characteristics of inhomogeneous and annular AgNOR as well as total number and area of all AgNOR yielded positive correlation with the cell and nucleus size, circumference and other morphometric characteristics analyzed. When compared with DNA histogram characteristics, the cell and nucleus morphometric characteristics also showed certain regular patterns. Larger cells and nuclei were associated with a higher percentage of cells containing >4N DNA. Inhomogeneous AgNOR, total area of all AgNOR, and ratio of total AgNOR area and nucleus were greater when the percentage of cells in the peak was lower. The number and area of inhomogeneous AgNOR correlated positively with the percentage of cell containing a greater content of DNA than peak cells, higher percentage of cells containing >4N DNA, and higher percentage of cells in the S-phase of the cell cycle. At the same time, greater area of all AgNOR correlated positively with the percentage of cells containing >4N DNA. Unlike inhomogeneous AgNOR, annular AgNOR and ratio of annular AgNOR and nucleus showed an inverse relation with the percentage of cells containing >4N DNA or percentage of cells in the S-phase of the cell cycle.

Like in the peripheral blood, in the bone marrow homogeneous AgNOR showed negative correlation with cellular and nuclear morphometric characteristics, whereas the characteristics of inhomogeneous and annular AgNOR as well as the number and area of all AgNOR correlated positively with the size, circumference and other cell and nucleus morphometric characteristics analyzed. DI and percentage of cells in the peak of DNA histogram showed an inverse relation with the cell and nucleus size. Larger cells and nuclei were associated with a higher percentage of cells containing higher DNA content than peak cells. As well as in a higher percentage of cells in the S-phase of the cell cycle. Comparison of AgNOR characteristics and DNA histogram characteristics revealed the percentage of cells containing lower DNA content than the diploid peak of the histogram to correlate positively with the number and area of homogeneous AgNOR. The higher the percentage of cells with DNA content greater than peak cells, the the larger was the area of all AgNOR. The number and area of inhomogeneous AgNOR correlated positively with the percentage of cells that also contained more DNA than those in the peak of DNA histogram, while yielding an inverse relationship with the percentage of cell in the peak.

Analysis of the morphometric characteristics of the lymph node lymphatic cells revealed only N/C ratio to show negative correlation with homogeneous AgNOR. Maximal cell radius yielded positive correlation with the area of inhomogeneous AgNOR, and the number and total area of all AgNOR. The lymph node lymphatic cells showed a considerably lower rate of correlations between DNA and AgNOR characteristics; only the total number and area of AgNOR were greater with a higher percentage of cells containing DNA >4N.

(b) In the subgroup of patients with typical and atypical CLL (B-CLL-TYP and B-CLL-ATYP)

In this subgroup, the percentage of cells with lower DNA content than peak cells correlated positively with the number of homogeneous AgNOR in bone marrow lymphatic cells. The characteristics of inhomogeneous AgNOR (number and area) showed an inverse correlation with the percentage of cells in the peak of DNA histogram, while yielding positive correlation with the percentage of cells with DNA content exceeding that in the peak cells or DNA content >4N.

A similar pattern was recorded for peripheral blood lymphatic cells: the lower the number of cells in the dip-

TABLE 1
SPEARMAN RANK CORRELATION BETWEEN MORPHOMETRIC CHARACTERISTICS, ARGYROPHILIC NUCLEOLAR ORGANIZATION REGION CHARACTERISTICS AND LYMPHATIC CELL DNA HISTOGRAM IN PERIPHERAL BLOOD OF LEUKEMIC LYMPHOPROLIFERATIVE DISEASE PATIENTS

Spearman Rank Order Correlations					
	PBAGAHOM/N	PBAGAINH	PBAGNAN	PBAGATOT	PBDNA%S>4N
PBMAC	–0.260286*	0.411878*	0.002588	0.520050*	0.268947*
PBMOC	–0.231187	0.354180*	0.012462	0.460307*	0.275398*
PBMMIRC	–0.368542*	0.448534*	0.020353	0.544974*	0.112562
PBMMARC	–0.182966	0.329715*	0.003340	0.442939*	0.251818
PBMCAC	–0.248888*	0.403110*	–0.007118	0.501597*	0.275175*
PBMLC	–0.182006	0.339860*	0.017222	0.462311*	0.263830
PBMBC	–0.286254*	0.430247*	–0.048555	0.483728*	0.262273
PBMFFC	–0.042981	0.215071	–0.202215	0.145684	–0.197539
PBMFFEC	0.059096	–0.104770	0.171132	0.027325	0.082753
PBMSUMAN	–0.279491*	0.393216*	0.040852	0.548648*	0.219784
PBMN/C	0.115332	–0.232022	–0.012692	–0.304874*	–0.238025
PBMAN	–0.278196*	0.402067*	0.040101	0.561257*	0.213556
PBMON	–0.236739	0.335393*	0.054212	0.474752*	0.208439
PBMMIRN	–0.303914*	0.431375*	–0.013318	0.568312*	0.100327
PBMMARN	–0.207181	0.328588*	0.046781	0.456925*	0.222009
PBMCAN	–0.269387*	0.386452*	0.045695	0.541008*	0.219784
PBMLN	–0.220708	0.342407*	0.052187	0.481641*	0.234021
PBMBN	–0.299113*	0.442396*	–0.016011	0.557040*	0.187528
PBMFFN	–0.092078	0.238409	–0.220167	0.190606	–0.082530
PBMFFIN	–0.022398	–0.020436	0.168168	0.042396	0.048050
PBDNADI	–0.085971	–0.027394	0.115021	0.181850	
PBDNA%CPIK	0.310507	–0.487430*	0.177307	–0.419512*	
PBDNA%C<PIK	–0.074488	–0.060322	0.086687	0.052160	
PBDNA%C>PIK	–0.085378	0.330534*	–0.184509	0.201435	
PBDNA%C>4N	0.053809	0.411988*	–0.282203	0.317514*	
PBDNA%SFC	–0.104367	0.321613*	–0.324771*	0.136893	

*Correlations significant at $p<0.05$ (marked in red), PB – peripheral blood, MAC – cell area (μm^2), MOC – cell outline, MIRC – cell minimum radius; MARC – cell maximum radius, MCAC – cell convex area (μm^2), MBC – cell breadth; MLC – cell length, MFFC – cell regularity (4π area/r^2), MFFEC – cell elongation (length/breadth), MAN – nuclear area (μm^2), MON – nuclear outline, MIRN – nuclear minimum radius, MARN – nuclear maximal radius, MCAN – nuclear convex area (μm^2), MLN – nuclear length, MBN – nuclear breadth, MFFN – nuclear regularity (4π area/r^2), MFFEN – nuclear elongation (length/breadth), AGAINHOM – area of inhomogeneous AgNOR (μm^2), AGNAN – number of annular AgNOR, AGATOT – total number of AgNOR, AGHOM/N – ratio of homogeneous AgNOR area and area of nucleus, DNADI – DNA index; DNA%CPIK – percentage of cells in peak of DNA histogram, DNA%C<PIK – percentage of cells with lower contents of DNA than cells in peak of DNA histogram, DNA%C>PIK – percentage of cells with higher contents of DNA than cells in peak of DNA histogram, DNA%C>4N – percentage of cells with higher contents DNA than 4N; DNA% CSFC – percentage of cells in S-phase

loid peak of DNA histogram, the higher was the number and area of inhomogeneous AgNOR. At the same time, the greater the number of cells with DNA content exceeding that in the peak cells or >4N, or the more cells in the S-phase of the cell cycle, the greater was the number and area of inhomogeneous AgNOR. The correlation of total area of all AgNOR and ratio of total area of all AgNOR/nucleus followed a similar pattern: these parameters were lower with the higher percentage of cells in PEAK and higher with the greater percentage of cells containing >4N DNA. Homogeneous AgNOR in the lymph node lymphatic cells had greater area when the percentage of cells containing less DNA than peak cells was higher, with greater ratio of homogeneous AgNOR/nucleus, and when fewer cells were in the S-phase of the cell cycle. Total number and area of all AgNOR, and the ratio of total area of all AgNOR/nucleus were greater when the percentage of cells with DNA content exceeding that in the peak cells was higher or when there were more cells with DNA content >4N.

Distribution of cells in different compartments of tumor mass

(a) Bone marrow and peripheral blood

The area (99.79±22.93 μm^2) and regularity of nuclei (0.84±0.02) in peripheral blood lymphatic cells were statistically significantly greater than in bone marrow lymphatic cells (area 90.62±16.70 μm^2 and regularity 0.82±0.02) in total CLLPD population (p=0.002273 for area and p=0.000275 for regularity) as well as in the B-CLL--TYP&ATYP subpopulation (p=0.005566 for area and p=0.000275 for regularity). The nucleus to cytoplasm ratio was lower in peripheral blood (CLLPD 0.70±0.06 and B-CLL-TYP&ATYP 0.71±0.06) than in bone marrow (CLLPD 0.74±0.04 and B-CLL-TYP&ATYP 0.74±0.03) and in both groups (larger lymphatic cell nuclei and lesser cytoplasm in bone marrow) (Table 2, Figure 2).

(b) Bone marrow and lymph node

In comparison with lymph node, bone marrow showed a statistically significantly lower number (0.31±0.19 for bone marrow and 0.56±0.33 for lymph node) and smaller total area of homogeneous AgNOR (0.26±0.16 for bone marrow and 0.44±0.26 for lymph node), and lower minimal, maximal and mean values of these parameters. Lymph node was associated with a lower number of annular AgNOR (lymph node 0.33±0.22 and bone marrow 0.46±0.24), greater number of total AgNOR (lymph node 1.55±0.32 and bone marrow 1.42±0.23), and lower cell percentage in the peak of DNA histogram (lymph node 32.80±10.90 and bone marrow 36.24±8.98) in total CLLPD population and B-CLL-TYP&ATYP subgroup (Table 3).

(c) Peripheral blood and lymph node

The difference of morphometric parameters of lymphatic cell area (CLLPD, p=0.000143 and B-CLL-TYP& ATYP, p=0.000627) and convexity of the cell surface (CLLPD, p=0.000005 and B-CLL-TYP&ATYP, p=0.000002) and nucleus were statistically significantly i.e. those parameters were lower in lymph node than in peripheral blood. The AgNOR characteristics, i.e. the number (CLLPD, p=0.000576 and B-CLL-TYP&ATYP, p=0.000243) and area of homogeneous AgNOR (CLLPD, p=0.000576 and B-CLL-TYP&ATYP, p=0.000246) and their ratio relative to the nucleus (CLLPD, p=0.002918 and B-CLL-TYP& ATYP, p=0.003708) were lower in peripheral blood. The number of annular AgNOR (CLLPD p=0.002943 and B-CLL-TYP&ATYP, p=0.003685), their total area (CLLPD, p=0.023458 and B-CLL-TYP&ATYP, p=0.033089) (Figure 3), and their minimal (CLLPD, p=0.026769 and B-CLL-TYP&ATYP, p=0.031885) and maximal area (CLLPD, p=0.023417 and B-CLL-TYP&ATYP, p=0.030083)

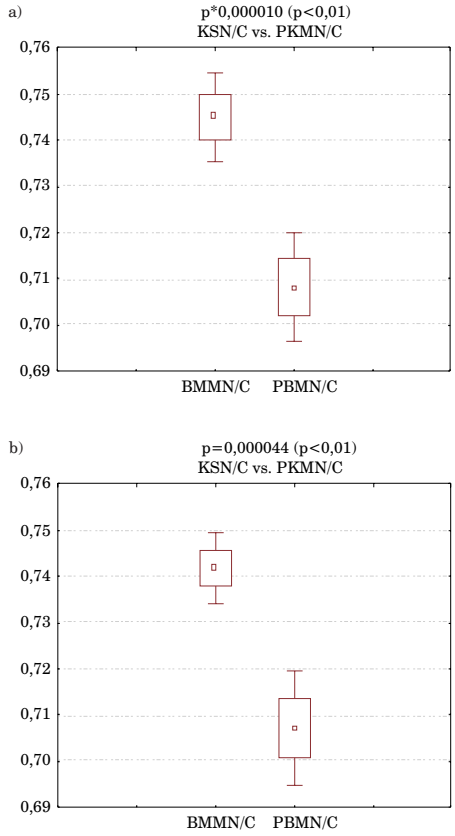

Fig. 2. a) Nucleo/cytoplasm ratio of bone marrow (BMMN/C) and peripheral blood (PBMN/C) lymphatic cells of patients with chronic leukemic lymphoproliferative disorders and b) B chronic lymphocytic leukemia; statistically significant, p<0.01.

TABLE 2
STATISTICALLY SIGNIFICANT DIFFERENCES IN IMAGE PARAMETERS OF BONE MARROW AND PERIPHERAL BLOOD LYMPHATIC CELLS

VARIABLE	CLLPD			B-CLL(T&A)		
	p	BM $\overline{X}$±SD	PB $\overline{X}$±SD	P	BM $\overline{X}$±SD	PB $\overline{X}$±SD
BMMAC # PBMAC (μm^2)	0.002273	90.62±16.70	99.79±22.93	0.005566	89.62±12.53	97.38±20.40
BMMFFJ # PBMFFN	0.000275	0.82±0.02	0.84±0.02	0.000275	0.83±0.03	0.84±0.03
BMMN/C # PBMN/C	0.000010	0.74±0.04	0.70±0.06	0.000044	0.74±0.03	0.71±0.06

$\overline{X}$ – mean, SD – standard deviation; p – probability of a statistically significant difference, NS – statistically nonsignificant; CLLPD – chronic leukemic lymphoproliferative disorders, B-CLL(T&A) – B chronic lymphocytic leukemia (typical and atypical), BM – bone marrow, PB – peripheral blood; LN – lymph node, MAC – cell area, MFFN – nuclear regularity (4p area/r^2), MN/C – nucleo-cytoplasmic ratio

TABLE 3
STATISTICALLY SIGNIFICANT DIFFERENCES IN IMAGE PARAMETERS OF BONE MARROW AND LYMPH NODE LYMPHATIC CELLS

VARIABLE	CLLPD			B-CLL(T&A)		
	P	BM $\overline{X}$±SD	LN $\overline{X}$±SD	P	BM $\overline{X}$±SD	LN $\overline{X}$±SD
BMAGNHOM # LNAGNHOM	0.000006	0.31±0.19	0.56±0.33	0.000014	0.31±0.17	0.56±0.34
BMAGAHOM # LNAGAHOM	0.000046	0.26±0.16	0.44±0.26	0.000042	0.26±0.14	0.45±0.26
BMAGNAN # LNAGNAN	0.006717	0.46±0.24	0.33±0.22	0.003040	0.48±0.22	0.33±0.22
BMAGNTOT # LNAGNTOR	0.015294	1.42±0.23	1.55±0.32	0.012914	1.43±0.16	1.54±0.35
BMDNA%CPEAK# KSDNA%CPEAK	0.010407	36.24±8.98	31.80±10.90	0.002434	37.56±7.76	32.08±11.36

$\overline{X}$ – mean, SD – standard deviation, p – probability of a statistically significant difference, NS – statistically nonsignificant, CLLPD – chronic leukemic lymphoproliferative disorders, B-CLL(T&A) – B chronic lymphocytic leukemia (typical and atypical), BM – bone marrow; PB – peripheral blood, LN – lymph node, AGNHOM – number of homogeneous AgNOR, AGAHOM – area of homogeneous AgNOR (mm^2), AGNAN – number of annular AgNOR, AGNTOT – total number of AgNOR, KSDNA%CPEAK – percentage of cells in peak of DNA histogram

were greater in peripheral blood lymphatic cells than in lymph node lymphatic cells (Table 4). Cell percentage in the peak of DNA histogram (CLLPD, p=0.009674 and B-CLL-TYP&ATYP, p=0.011876) (Figure 4) was lower in lymph node as compared with peripheral blood in both study groups.

Discussion and Conclusion

Morphometric characteristics of the cells and/or nuclei, DNA content and AgNOR characteristics have been analyzed in various benign[19,20], premalignant and malignant lesions of different organs[21], including leukemias

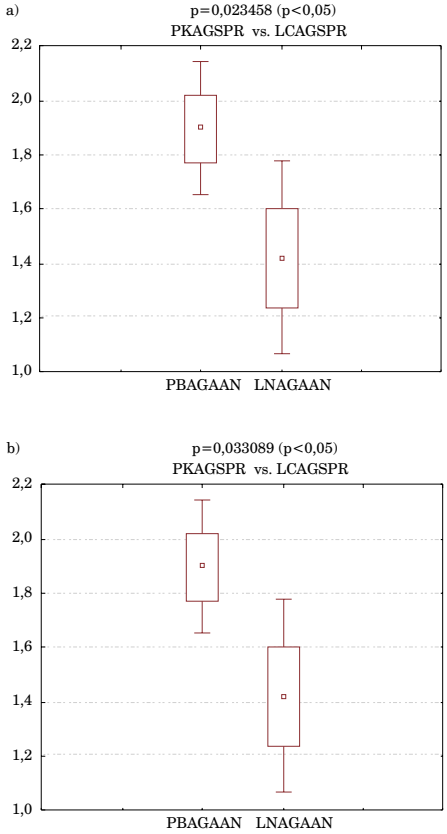

Fig. 3. a) Area of annular AgNOR (mm^2) of peripheral blood (PBAGAAN) and lymph node (LNAGAAN) lymphatic cells of patients with chronic leukemic lymphoproliferative disorders and b) B chronic lymphocytic leukemia; statistically significant, p<0.05.

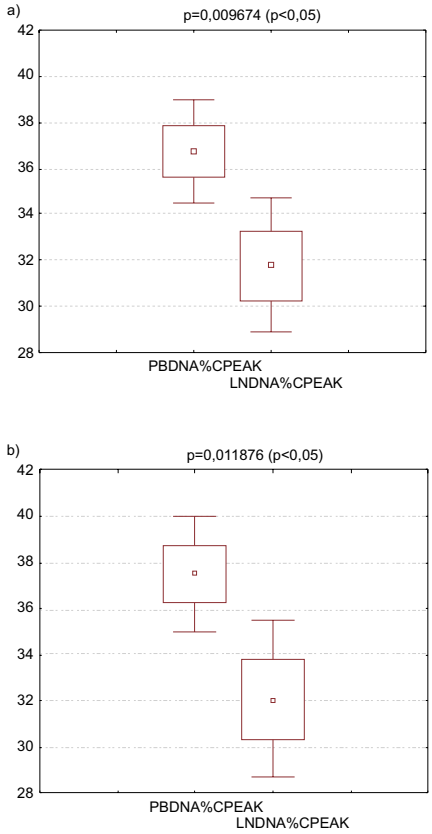

Fig. 4. a) Percentage of cells in peak of DNA histogram of peripheral blood (PBDNA%CPEAK) and lymph node (LNDNA% CPEAK) lymphatic cells of patients with chronic leukemic lymphoproliferative disorders and b) B chronic lymphocytic leukemia; statistically significant, p<0.05.

TABLE 4
STATISTICALLY SIGNIFICANT DIFFERENCES IN IMAGE PARAMETERS OF PERIPHERAL BLOOD AND LYMPH NODE LYMPHATIC CELLS OBTAINED BY IMAGE COMPUTER ANALYSIS

VARIABLE	CLLPD			B-CLL(T&A)		
	P	PB $\overline{X}$±SD	LN $\overline{X}$±SD	P	PB $\overline{X}$±SD	LN $\overline{X}$±SD
PBMAC # LNMAC (mm^2)	0.000143	99.79±22.93	86.10±15.35	0.000627	97.38±20.40	85.73±11.90
PBMCAC # LNMCAC (mm^2)	0.000005	102.56±24.37	88.22±15.92	0.000002	100.06±22.01	87.75±12.27
PBAGBNOM # LNAGNHOM	0.000576	0.31±0.33	0.56±0.33	0.000243	0.29±0.20	0.56±0.34
PBAGAHOM # LNAGAHOM (mm^2)	0.000576	0.28±0.22	0.44±0.26	0.000246	0.27±0.20	0.45±0.26
PBAGHOM/N #LNAGHOM/N	0.002918	0.004±0.003	0.01±0.02	0.003708	0.004±0.003	0.01±0.03
PBAGNAN # LNAGNAN	0.002943	0.48±0.26	0.33±0.22	0.003685	0.49±0.27	0.30±0.22
PBAGAAN # LNAGAAN (mm^2)	0.023458	1.90±0.26	1.44±1.13	0.033089	1.90±1.07	1.42±1.08
PBAGMAN # LNAGMAN (mm^2)	0.023458	1.93±1.11	1.36±1.08	0.045500	1.90±0.97	1.33±1.01
PBAGMIAN # LNAGMIAN (mm^2)	0.026769	1.79±1.01	1.35±1.07	0.031885	1.75±0.96	1.32±1.00
PBAGMAAN # LNAGMAAN (mm^2)	0.023417	1.84±1.04	1.38±1.08	0.030083	1.80±0.99	1.35±1.02
PBDNA%CPEAK # LNDNA%CPEAK	0.009674	36.75±10.47	31.80±10.90	0.011876	37.50±10.75	32.08±11.36

$\overline{X}$ – mean, SD – standard deviation, p – probability of a statistically significant, NS – statistically nonsignificant, CLLPD – chronic leukemic lymphoproliferative disorders, B-CLL(T&A) – B chronic lymphocytic leukemia (typical and atypical), BM – bone marrow, PB – peripheral blood, LN – lymph node, MAC – cell area (mm^2), MCAC – cell convex area (mm^2), AGNHOM – number of homogeneous AgNOR, AGAHOM – area of homogeneous AgNOR (mm^2), AGHOM/N – ratio of homogeneous AgNOR area and area of nucleus, AGNAN – number of annular AgNOR; AGAAN – area of annular AgNOR (mm^2), AGSMAN – average area of annular AgNOR (mm^2), AGMIAN – minimum area of annular AgNOR (mm^2), AGMAAN – maximum area of annular AgNOR (mm^2), KSDNA%CPEAK – percentage of cells in peak of DNA histogram

and lymphomas[22–25]. In contrast to most studies that used some of the quantitative morphometric methods[26], AgNOR analysis[25] and ICM[27], Böcking et al.[28] employed multimodal cell analysis that included nucleus morphometry, chromatin structure, nucleolar characteristics, AgNOR and DNA-ICM, in order to upgrade diagnostic accuracy of malignant tumors through analysis of samples with a small number of atypical cells and thus improving the early diagnosis of those diseases. The relation of AgNOR number and ploidy remains a controversial issue[29,30]. Some studies failed to demonstrate correlation between AgNOR parameters and DNA parameters[31]. However, analysis of the relation of AgNOR aggregates and cell proliferation in general, not only in the diagnosis of malignancy, has shown that the number of AgNOR aggregates per nucleus does not correlate well with higher proliferative activity, while the size of these aggregates was consistently related to the cell proliferation[32]. Our results demonstrated the correlation among morphometric characteristics, proliferative characteristics of different AgNOR types, and newly described features of the diploid type of histogram. Based on these, the following conclusions were drawn: cells with low proliferative activity are those that have small homogeneous AgNOR and most cells in the peak of DNA histogram; highly proliferative cells have inhomogeneous AgNOR and most of them have DNA content higher than peak cells, or there are pathologic mitoses (DNA >4N), or a greater number of cells are in the S-phase of the cell cycle. Cells with medium sized annular AgNOR are somewhere in--between.

Previous studies of lymphatic cells were generally performed in a single tumor mass compartment, mostly on peripheral blood smear[33,34] or lymph node[35]. Jakšić et al.[4] were the first to analyze the relationship of adhesion molecules as major factors in tumor mass distribution among particular tumor mass compartments. In the present study, morphometric and kinetic characteristics of tumor cells were analyzed for the first time, and compared among different tumor mass compartments: bone marrow, peripheral blood and lymph nodes in the same patient. In these compartments, neoplastic cells are found in different microenvironments. Our results indicated that lymphatic cells with affinity for accumulation in bone marrow as a rule had low proliferative activity (the highest percentage of cells in the peak of G_0/G_1 phase). The cells performing affinity to accumulate in lymph nodes, migrated to lymph nodes (higher number of total AgNOR, higher area of proliferative inhomogeneous AgNOR and lowest percentage of cells in the G_0/G_1 phase). Cell migration from bone marrow to lymph nodes and between lymph nodes takes place through the peripheral blood (where a mixture of cells with lower and higher proliferative activity was found; a higher percentage of cells in synthetic phase of cell (SFC) and concurrently in the G_0/G_1 phase of the cell cycle; larger cells with smaller nuclei with annular AgNOR of the intermediary grade of proliferation). Peripheral blood appeared to have just a transport role.

Analysis of the cell size and proliferative activity in different tumor mass compartments indicated a regular pattern in both total CLLPD population and B- CLL-

-TYP&ATYP subgroup. Whereas bone marrow and peripheral blood cells did not differ substantially according to size and proliferative activity, comparison of peripheral blood and lymph node cells produced different results. To the contrary of what was expected, as small cells are more indolent and larger ones more proliferative, the analysis showed the cells in peripheral blood to be the largest and most indolent, in contrast to lymph node cells that were smallest and most active. The analysis included routine slides, posing the question of various preparation techniques employed for lymph node, bone marrow and peripheral blood FNA smears. The results obtained can be confirmed or challenged by measuring cells in native sample. Although the great majority of statistically significant parameters compared were recorded in peripheral blood, a peripheral blood sample alone is neither representative nor adequate to study proliferative activity or disease stability, thus posing the question of careful evaluation of these parameters in all tumor mass compartments[36].

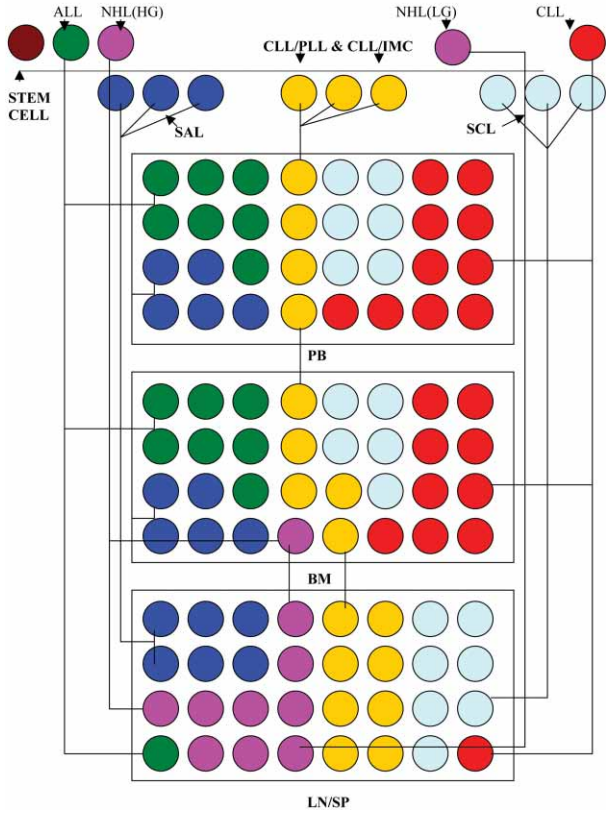

Fig. 5. Hypothesis of »single« and »multiple« programmed stops in the development of chronic lymphoproliferative diseases: PB – peripheral blood; BM – bone marrow; LN/SP – lymph node/spleen; ALL (typical acute lymphatic leukemia) – »single programmed stop« at an earlier stage of development; NHL (typical Non-Hodgkin lymphoma) – »single programmed stop« also at an earlier stage of development; SAL (subacute leukemia) – »multiple programmed stops» at an earlier stage of development; CLL (typical chronic lymphocytic leukemia) – »single programmed stop« at a high stage of differentiation; SCL (subchronic leukemia) – »multiple programmed stops« at a higher stage of differentiation; CLL/PLL, CLL/IMC (variant of chronic lymphocytic leukemia) – »single or multiple programmed stops« at the middle of the differentiation process, ALL – acute lymphoblastic leukemia, NHL (HG) – non-Hodgkin lymphoma (high grade), NHL (LG) – non-Hodgkin lymphoma (low grade)

The question is whether our results could be considered in explaining the hypothesis of »single« and »multiple stops« to elucidate the pathogenesis of typical leukemias and lymphomas as well as various leukemic forms of chronic lymphoproliferative diseases (Figure 5). Lymphoproliferative disorders develop because of deranged differentiation of lymphatic cells and the consequent accumulation of long-living functionally inactive neoplastic B-lymphocytes arrested in the G_0 phase of the cell cycle[37,38]. Dysregulation of differentiation occurs early in the bone marrow, and these »damaged« cells continue to proliferate and differentiate in the primary (bone marrow) and secondary lymphatic organs (lymph nodes, spleen, etc.), imitating the normal course of reactive cell development to the moment of »programmed stop« at which point accumulation of lymphatic cells of a more mature or immature morphological appearance occurs. The functional inactivity of pathological cells and immunodeficiency result in a vicious circle, i.e. stimulus to the bone marrow to direct stem cells to immunocompetent cells, which, due to unregulated differentiation, cannot differentiate into functionally active B- and/or T-lymphocytes and satisfy the peripheral demand. The accumulation of pathological cells in the lymph nodes, bone marrow and/or peripheral blood leads to the development of lymphoproliferative diseases (leukemia and lymphoma). Which mechanisms are responsible for derangement of differentiation and the consequent accumulation of pathological cells? Demonstration of the BCL2, BCL6 and IGV genes and their products has shown that malignomas with or without mutated genes belong to different prognostic groups, heterogeneous in morphological and clinical features. As an example serve separate groups of diffuse large B-cell lymphoma (DLBCL) classified according to biological characteristics of the tumor by the corresponding degree of B-cell differentiation. Two major DLBCL subtypes have been defined, one with the presence of germinal-center B-cell gene expression (B-cell-like profile, GCB) and the other with low expression of B-cell germinal-center genes and expression of normally activated B-cell genes (activated B-cell-like profile, ABC). There is a significant difference in survival between these two DLBCL groups. Will the discovery of different gene mutations (one or more mutations) for each derangement in differentiation follow in the future? Are somatic gene mutations alone accountable for different morphological and clinical features? According to the currently adopted WHO classification, certain instances of leukemic forms and localized diseases are considered to be different manifestations of the same disease (B-lymphoblastic leukemia/B-lymphoblastic lymphoma on the one hand and B-chronic lymphocytic leukemia/B-lymphocytic lymphoma of small cells on the other hand). Does the binding of adhesion molecules as major factors in the distribution of

tumor mass among particular tumor compartments play a role in clinical manifestation of the disease[4]?

Are, therefore, leukemias and lymphomas a single disease entity with different clinical manifestations as the result of different tumor mass distribution in various compartments (lymphoid organs, bone marrow and peripheral blood)? Is it more appropriate to consider them separate diseases developing due to dysregulated differentiation, which can occur at any particular stage (at single or multiple points), where different points of »stop« result in heterogeneous morphology and affinity towards accumulation in the bone marrow, peripheral blood and lymph node (also taking into account the issue of microenvironment). These cases should not be included in the same therapeutic trials as B-CLL.

The hypothesis is that a »programmed stop« occurs at various stages of differentiation (but at a single point), smaller or larger lymphatic cells infiltrate the lymph node, and the clinical presentation of a malignant lymphoma develops (small cell or large cell type of lymphoma, depending on the point of »stop« and stage of differentiation and maturation).

In case when the »stop« occurs at a later stage of differentiation and maturation, also at a single point, the small indolent lymphocytes accumulate in the bone marrow and peripheral blood and lead to clinical presentation of typical chronic lymphocytic leukemia.

Differentiation arrested between the two extremes (at one and/or more points) leads to the accumulation of medium-sized lymphatic cells which have a high migration potential resulting in clinical presentation of B-prolymphocytic leukemia and variants of chronic lymphocytic leukemia (chronic lymphocytic/prolymphocytic leukemia or chronic lymphocytic leukemia with lymphoplasmacytoid differentiation).

However, since in the oncogenesis of lymphatic cells the »programmed stop« never occurs at a single point but rather multiple damages are possible, it is obvious that there are transitions between the three typical forms of the disease, as well as morphological and proliferative variations in different tumor mass compartments, and those results in clinical heterogeneity.

»Multiple stops« shifted towards a high stage of differentiation are the cause of accumulation of well differentiated indolent cells in the bone marrow and proliferative cells with a predilection for the lymph node, resulting in clinical presentation of subchronic leukemias (SCL).

»Multiple stops« at an earlier stage of differentiation result in the accumulation of highly proliferative cells with a predilection for lymph node and more indolent cells with migration potential, leading to clinical presentation of subacute leukemias (SAL).

Both subtypes (subacute and subchronic leukemias) can be divided into TYPE A – leukemic form with atypical lymphatic cell counts of $>5\times10^9$/L in peripheral blood with infiltration of the bone marrow and lymph nodes, and TYPE B – subleukemic form with lymph node enlargement, bone marrow infiltration >40% but less than 5×10^9/L of malignant cells in peripheral blood. Additional genomic studies are warranted to establish their precise nature[39].

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MULTIMODALNA STANIČNA ANALIZA U KRONIČNIM LEUKEMIJSKIM LIMFOPROLIFERATIVNIM BOLESTIMA I HIPOTEZA »JEDNOSTRUKIH« I »VIŠESTRUKIH« PROGRAMIRANIH STOPOVA U NASTANKU TIPIČNIH I ATIPIČNIH OBLIKA LEUKEMIJA I LIMFOMA

S A Ž E T A K

Studija je obuhvatila morfometrijsku analizu, analizu osobina regije nukleolarne organizacije (AgNOR) te statičku DNA citometriju u različitim odjeljcima tumorske mase: koštanoj srži (KS), perifernoj krvi (PK) i limfnim čvorovima (LČ) u bolesnika s kroničnim leukemijskim limfoproliferativnim bolestima. Ukupno je analizirano 71895 stanica, na osobnom računalu »SFORM« tvrtke VAMSTEC, Zagreb. Međusobnom analizom morfometrijskih, proliferacijskih i kinetičkih pokazatelja vidjelo se kako nisko proliferativne stanice imaju male homogene AgNOR-e te većinu stanica u vršku DNA histograma. Visoko proliferativne stanice su s inhomogenim AgNOR-ima, od kojih većina sadrži količinu DNA veću od stanica u vršku; patološke mitoze (DNA>4N) ili veći broj stanica u S-fazi staničnog ciklusa. Negdje u sredini su srednje proliferativne stanice s prstenastim AgNOR-ima. Analizom u različitim odjeljcima tumorske mase vidljivo je kako limfatične stanice koje imaju afinitet prema akumulaciji u KS, u pravilu imaju malu proliferativnu aktivnost (najveći postotak stanica u vršku G_o/G_1 faze). Stanice s afinitetom prema akumulaciji u LČ imaju veći broj ukupnih AgNOR-a, veću površinu proliferativnijih, inhomogenih AgNOR-a i najmanji postotak stanica u G_o/G_1 fazi. Sama migracija stanica iz KS prema LČ kao i između LČ događa se u PK gdje su prisutne stanice s nižom i višom proliferativnom aktivnosti (viši postotak stanica u S-fazi i istodobno u G_o/G_1 fazi staničnog ciklusa kao i prisustvo većih stanica, ali manjih jezgara, s prstenastim AgNOR-ima intermedijalnog stupnja proliferativnosti). Izgleda kako PK ima samo transportnu ulogu. Analizirajući veličinu stanica i njihovu proliferativnu aktivnost u različitim odjeljcima tumorske mase uočeno je da se stanice u KS i PK ne razlikuju bitno po veličini i proliferativnoj aktivnosti, dok je obrnuta situacija ako se gleda PK i LČ. Nasuprot očekivanju, kako su male stanice mirnije, a veće proliferativnije, analiza je pokazala da su u PK stanice najveće i najmirnije, za razliku od LČ gdje su najmanje i najaktivnije. Postavljena je hipoteza jednostrukih i višestrukih »programiranih stopova» u nastanku tipičnih oblika leukemija i limfoma te subakutnih i subkroničnih leukemija. Poremećaj diferencijacije može nastati na bilo kojem stupnju, a različito mjesto »stopa« rezultira različitom morfologijom te različitim afinitetom prema akumulaciji u KS, PK I LČ.