

New Parameters of Diploid Histogram of Image DNA Cytometry and Newly Characterized Types of Nucleolar Organizer Region Structures in Defining the Proliferative-Kinetic Index in Chronic Leukemic Lymphoproliferative Disorders

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OBJECTIVE: To introduce new parameters of diploid histogram of image DNA cytometry and new types of silver-stained nucleolar organizer regions (AgNORs) and to validate resulting proliferative-kinetic index (PKI) in a prognostic study of patients with chronic leukemic lymphoproliferative disorders (CLLPD).

STUDY DESIGN: A total of 413 smears of from various tumor mass compartments—bone marrow, peripheral blood and lymph node—were analyzed in CLLPD as a whole, as well as separately in the B-chronic lymphocytic leukemia with variants (B-CLL+V). The analysis of the diploid histogram included percentage of cells at the peak

of the DNA histogram and percentage of cells with lower and higher contents of DNA than cells at the peak. The new types of AgNORs were described as homogeneous, inhomogeneous and annular.

RESULTS: The newly introduced parameters of DNA and AgNOR are significant predictors of survival. Based on the most representative AgNOR and DNA characteristics related to survival, the PKI score was calculated. The CLLPD and B-CLL+V patients had a statistically significantly better prognosis when PKI was < 4.

CONCLUSION: PKIs have confirmed the hypothesis that different prognostic subgroups could be identified

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within the homogeneous groups of neoplasms with relatively low malignancy (CLLPD and B-CLL+V). (Anal Quant Cytol Histol 2009;31:313–323)

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The chronic leukemic lymphoproliferative disorders (CLLPD) are heterogeneous, disseminated forms of mature B- or T-lymphocytic neoplasms, with variable clinical features.^{1,2} The majority of patients with CLLPD have chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL). Of the remaining non-CLL CLLPD cases, most are due to leukemic phases of lymphomas, including hairy cell leukemia (HCL) or prolymphocytic leukemia (PLL). However, in a small number of patients, no definitive diagnosis can be made (unclassified CLLPD).³

A typical B-CLL shows the morphology of small mature lymphocytes in peripheral blood, bone marrow and lymph nodes; expresses CD19, CD5 and CD23 antigens⁴; and is marked by low proliferative activity.⁵ Atypical CLL (variants of CLL) represents ~15% of CLL cases and comprises 2 major subtypes: CLL/PLL and CLL with lymphoplasmacytoid differentiation. CLL/PLL is defined by >10% but <55% of prolymphocytes in peripheral blood. The cases that show lymphoplasmacytoid morphology are called atypical CLL with lymphoplasmacytoid differentiation.⁶

Image DNA Cytometry

Tumor genesis is often associated with chromosomal abnormalities, changes of the genome and measurable differences in DNA contents (i.e., ploidy).⁷ DNA contents can be measured by flow cytometry or image DNA cytometry (ICM). For ICM, 100–300 cells per sample suffice, and a highly representative DNA histogram is obtained with direct cell visualization and observer selection of individual cells.⁸ Cytologic analysis is preferable to histology due to the involvement of the whole nuclei with ICM.⁹ The results of DNA measurement in tumor cells by ICM are expressed as relative values in relation to the reference cells, in a manner as efficacious on archived specimens as it is on fresh specimens.¹⁰ The reference cells which contain diploid amounts of DNA are lymphocytes (that are, however, obviously not appropriate for this study), granu-

locytes and fibroblasts. Ploidy is determined based on the ratio between the integrated optical density (IOD) of the investigated nuclei and the reference nuclei as expressed by the DNA index (DI). Tumors are considered diploid if their DI value is 0.95–1.05 (according to Auer et al¹¹) or is between 0.9 and 1.5 (according to van Velthoven et al¹²).

Silver-Stained Nucleolar Organizer Regions

Silver-stained nucleolar organizer regions (AgNORs) are loops of ribosomal DNA that are situated on the short arms of acrocentric chromosomes. They are substructures of the nucleoli, although they can also be found outside the nucleoli. AgNORs are presented by binding silver (Ag) to intranuclear nonhistonic groups in the form of black dots within the interphase nucleus (hence the term AgNORs)¹³ on fresh and archived cytologic or histologic specimens.¹⁴ The linear correlation between the number of AgNORs and Ki-67 proliferating antigen¹⁵ expression and between the number of AgNORs and the percentage of cells in the synthesis (S) phase of the cell cycle has been found to be an indicator of cell proliferation.¹⁶ Some authors claim that these 2 correlations have both diagnostic and prognostic values.^{17,18}

Materials and Methods

Our study included 155 patients with CLLPD, rated according to the World Health Organization classification. In addition to the patients who represented the B-CLL with variants (B-CLL+V) (n = 136), the CLLPD population also included the patients diagnosed as the leukemic phase (n = 18) of follicle center lymphoma, HCL, splenic marginal zone lymphoma, mantle cell lymphoma or T lymphoproliferative disorders. In addition to the total CLLPD population, the B-CLL+V was analyzed separately. DNA and AgNOR analyses were performed on cytologic smears, for the first time on all 3 tumor mass compartments (bone marrow, peripheral blood and lymph nodes), on a SFORM PC (Vamstec, Zagreb, Croatia). The quantitative criterion for inclusion was the infiltration of >40% of lymphatic cells into the bone marrow with an absolute lymphocytosis in peripheral blood of $>5 \times 10^9/L$. Fresh smears and previously stained archival smears were stained using the Feulgen method¹⁹ and silver impregnation for ICM and AgNOR, respectively.¹³

For DNA, we used our newly developed method of analysis of the diploid histogram (Figure 1). The percentage of cells in the peak of the DNA his-

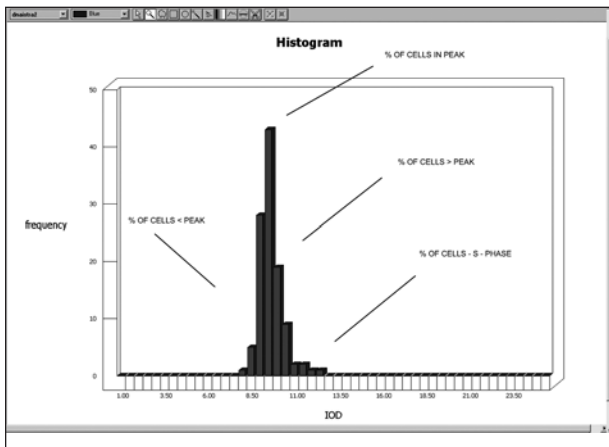


Figure 1 The new parameters of analysis of diploid DNA histogram.

ogram and the percentage of cells with lower and higher contents of DNA than cells in the peak of the DNA histogram were calculated. The percentage of cells in the S-phase and the percentage of cells that were $>4N$ were evaluated separately. According to Cornelisse et al,²⁰ the cut-off value of the G₀/G₁ phase on the right side of the histogram was evaluated by the overlap of the left half of G₀/G₁ peak around the modal value. After that, the proportion of cells with $\leq 4N$ amounts of DNA was considered the S-phase. Each histogram was presented with 50 classes of IOD values. For each sample the DNA index²¹ and coefficient of variation of reference cells, which should not exceed 6%,²² was calculated. We analyzed an average of 100 nuclei for Ag-NORs and between 120 and 250 nuclei for ICM.

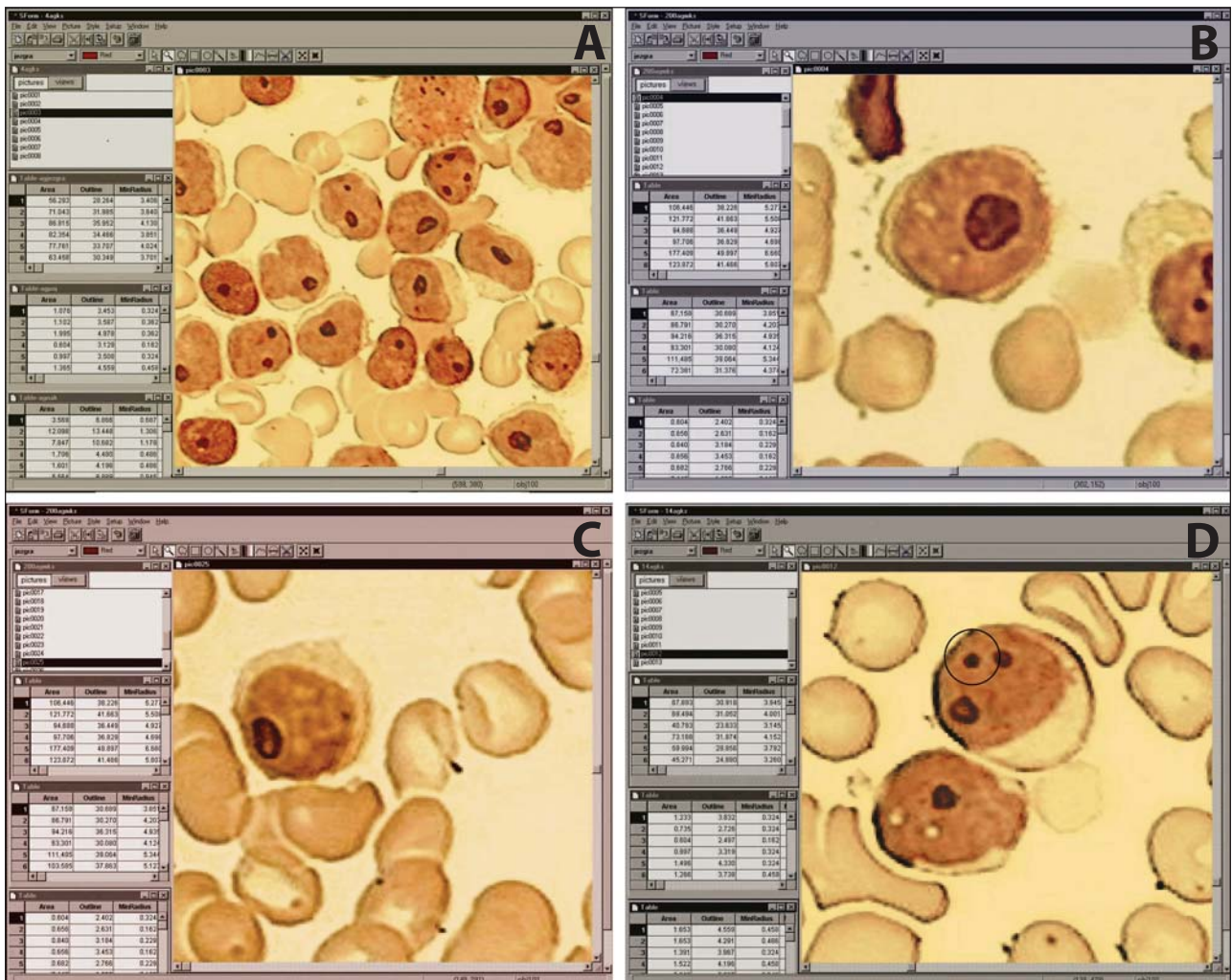


Figure 2 Types of AgNORs. (A) Overview-picture, (B) inhomogeneous, (C) anular and (D) homogeneous (in the circle).

Table 1 Statistically Significant Differences of AgNOR and ICM and Survival in Patients with CLLPD and B-CLL+V

Variable	CLLPD				B-CLL+V			
	Category	No. of patients (%)	Median survival (mo)	p Value	Category	No. of patients (%)	Median survival (mo)	p Value
BMDNA%CPEAK	≤47	77 (89.5)	91	0.03766	<47	66 (89.2)	91	0.03489
	>47	9 (10.5)	71		>47	8 (10.8)	48	
BMDNA%C>PEAK	≤55	80 (93.0)	88	0.04616	<55	80 (93.0)	88	0.04616
	>55	6 (7.0)	19		>55	6 (7.0)	19	
BMDNA%C<PEAK	>20	30 (37.0)	70	0.04769	≤20	27 (38.0)		0.03175
	≤20	51 (63.0)	88		>20	44 (62.0)		
BMDNA%CSFC	>4.1	40 (46.5)	106	0.02242	>4.1	39 (52.7)	106	0.02242
	≤4.1	46 (53.5)	85		<4.1	35 (47.3)	88	
PBDNADI	≤1.01	65 (81.3)	70	0.00553	<1.08	56 (78.9)	70	0.04918
	>1.01	15 (18.7)	250		>1.08	15 (21.1)	250	
PBDNA%C>PEAK	>45	30 (37.0)	70	0.04769	>45	27 (38.0)	70	0.03175
	≤45	51 (63.0)	88		<45	44 (62.0)	80	
LNDNA%CPEAK	>35	14 (27.5)	125	0.04806				NS
	≤35	37 (72.5)	55					
LNDNA%C>4N	≤2.5	45 (88.2)	88	0.03533				NS
	>2.5	6 (11.8)	19					
BMAGNHOM	>0.25	31 (49.2)	73	0.04749				NS
	≤0.25	32 (50.8)	87					
BMAGAHOM (μm ²)	>0.43	16 (25.3)	48	0.04218				NS
	≤0.43	47 (74.7)	91					
BMAGAHOM/N	<0.004	33 (52.4)	117	0.02621	≤0.004	27 (48.1)	117	0.01294
	>0.004	30 (47.6)	73		>0.004	25 (51.9)	73	
BMAGAINH (μm ²)	≤3.5	54 (85.8)	117	0.04365				NS
	>3.5	9 (14.2)	88					
BMAGATOT (μm ²)	≤6.4	55 (88.7)	88	0.04526	≤4	22 (41.5)	73	0.04104
	>6.4	7 (11.3)	31		>4	31 (58.5)	117	
BMAGNTOT	>1.6	9 (14.5)	34	0.04380	>1.65	13 (24.5)	34	0.04351
	≤1.6	53 (85.5)	88		≤1.65	40 (75.5)	91	
BMAGATOT/N	<0.07	46 (73.0)	88	0.04526	≤0.06	15 (28.3)	73	0.03822
	>0.07	17 (17.0)	34		>0.06	38 (71.7)	117	
PBAGNHOM	>0.3	32 (39.0)	117	0.02273	>0.3	27 (38.0)	117	0.04422
	≤0.3	50 (61.0)	73		≤0.3	44 (62.0)	85	
PBAGAHOM (μm ²)	>0.3	29 (35.3)	117	0.02803	>0.3	25 (35.2)	117	0.03140
	≤0.3	53 (64.7)	74		≤0.3	46 (64.8)	85	
PBAGAHOM/N	>0.002	59 (72.0)	88	0.00653				NS
	≤0.002	23 (28.0)	50					
PBAGAINH (μm ²)	<3	57 (69.6)	88	0.01548	≤3.1	55 (77.5)	88	0.04903
	>3	25 (30.4)	51		>3.1	16 (22.5)	63	
PBAGMAINH (μm ²)	≤3	64 (78.0)	88	0.00051	≤3.1	59 (83.1)	88	0.03386
	>3	18 (22)	31		>3.1	12 (16.9)	63	
PBAGAINH/N	≤0.04	55 (67.1)	88	0.04341	≤0.047	61 (85.9)	88	0.04840
	>0.04	27 (32.9)	56		>0.047	10 (14.1)	74	
PBAGNAN	≤0.65	57 (69.6)	88	0.04364	>0.65	49 (69.0)	250	0.03508
	>0.65	25 (30.4)	68		>0.65	22 (31.0)	68	
PBAGAAN (μm ²)	≤2.3	55 (67.1)	250	0.04937				NS
	>2.3	27 (32.9)	60					
PBAGMAAN (μm ²)	≤3	69 (88.5)	88	0.02348	≤3	62 (82.7)	117	0.04557
	>3	9 (11.5)	27		>3	13 (17.3)	27	
PBAGNTOT	>1.37	41 (50.0)	117	0.00653				NS
	≤1.37	41 (50.0)	73					

Granulocytes (N≥20) were used as reference cells for ICM.

Our own 3-type classification of the AgNOR

structure was used (Figure 2)—homogeneous type (within which no disaggregates were observed, even after magnification), inhomogeneous type (ir-

Table 1 *Cont'd*

Variable	CLLPD				B-CLL+V			
	Category	No. of patients (%)	Median survival (mo)	p Value	Category	No. of patients (%)	Median survival (mo)	p Value
PBAGATOT (μm^2)	≤ 6.5	72 (87.8)	88	0.00666	≤ 5	57 (80.3)	88	0.04690
	> 6.5	10 (12.2)	21		> 5	14 (19.7)	56	
PBAGATOT/N	> 0.07	24 (29.3)	21	0.01281	> 0.07	18 (25.4)	8	0.03224
	≤ 0.07	58 (70.7)	88		≤ 0.07	53 (74.6)	88	
LNAGAINH (μm^2)	≤ 3.674	33 (80.5)	88	0.02131				NS
	> 3.674	8 (19.5)	31					
LNAGATOT/N	> 0.065	23 (56.1)	70	0.04284				NS
	≤ 0.065	18 (43.9)	91					

AGAAN = area of anular AgNOR (μm^2), AGAHOM = area of homogeneous AgNOR (μm^2), AGAINH = area of inhomogeneous AgNOR (μm^2), AGAINH/N = ratio of inhomogeneous AgNOR area and area of nucleus, AGAMAAN = the largest area of anular AgNOR (μm^2), AGAMAINH = the largest area of inhomogeneous AgNOR (μm^2), AGATOT = total area of AgNOR (μm^2), AGATOT/N = ratio of total area of AgNOR and area of nucleus, AGHOM/N = ratio of homogeneous AgNOR area and area of nucleus, AGNAN = number of anular AgNOR, AGNHOM = number of homogeneous AgNOR, AGNINH = number of inhomogeneous AgNOR, AGNTOT = total number of AgNOR, BM = bone marrow, DNA%*C*PEAK = percentage of cells in peak of DNA histogram, DNA%*C*>PEAK = percentage of cells with higher contents DNA than cells in peak of DNA histogram, DNA%*C*<PEAK = percentage of cells with lower contents of DNA than cells in peak of DNA histogram, DNA%*C*SCF = percentage of cells in S-phase, DNA%*C*>4N = percentage of cells with higher contents DNA than 4N, LN = lymph node, PB = peripheral blood.

regularly disaggregated structures) and anular type (specific ring-shape disaggregation). For each type of AgNOR structure, the number, area (minimum, maximum and mean) and their proportion in relation to the nuclear surface; total number of all AgNORs; total area (minimum, maximum and mean); and the proportion of total area of all AgNORs in relation to the nuclear surface were evaluated. The Kaplan-Meier method was used for the analysis of survival (Statistica 7.1, StatSoft Inc., Tulsa, Oklahoma, U.S.A.).

Results

The total CLLPD population had a median age of 62 years. The oldest patients were in the B-CLL+V subgroup, with a median age of 63 years, and the youngest were in the non-B-CLL+V subgroup, with a median of 46 years ($p = 0.000111$), which was statistically significant. The study comprised 90 males and 65 females; the difference between male and female ages was not statistically significantly different ($p = 0.213349$). The results of survival in relation to the characteristics of AgNORs and ICM are tabulated in Table I.

ICM

Patients with CLLPD had a better prognosis if their bone marrow contained a percentage of cells in the peak between 28.2% and 47% (Figure 3A) or <55% of cells with greater DNA contents than cells in the peak (Table I). In patients with better survival

>4.1% of cells were in the S-phase of the cell cycle. In peripheral blood, if the DI was >1.01 with <45% of cells with higher DNA contents than cells in the peak, the group was considered prognostically favorable. In this case, the lymph node also contained >35% of cells in the peak and <2.5% of cells with the DNA contents that were >4N.

Poorer prognosis was associated with patients with B-CLL+V whose bone marrow had <37% or >47% of cells in the peak (Figure 3B). These patients also had >55% of cells with higher DNA contents than cells in the peak and <4.1% of cells in the S-phase of the cell cycle. In these cases, the DI was <1.08 in peripheral blood, and we observed >45% of cells with higher DNA content than cells in the peak. Better survival was seen in patients in whom proliferative activity in the bone marrow was greater—that is, if <47% of lymphatic cells were in the peak (but not <28.2%) and if >4.1% of cells were in the S-phase of the cell cycle.

AgNORs

The prognostic indicators in the bone marrow of patients with CLLPD that have been shown to be favorable include the number of homogeneous AgNORs being <0.25, the area of homogeneous AgNORs being <0.43 μm^2 , the proportion of the area of homogeneous AgNORs in relation to the nuclear surface being <0.004 and the area of inhomogeneous AgNORs being <3.5 μm^2 . Also, the factors associated with a better prognosis are the total area

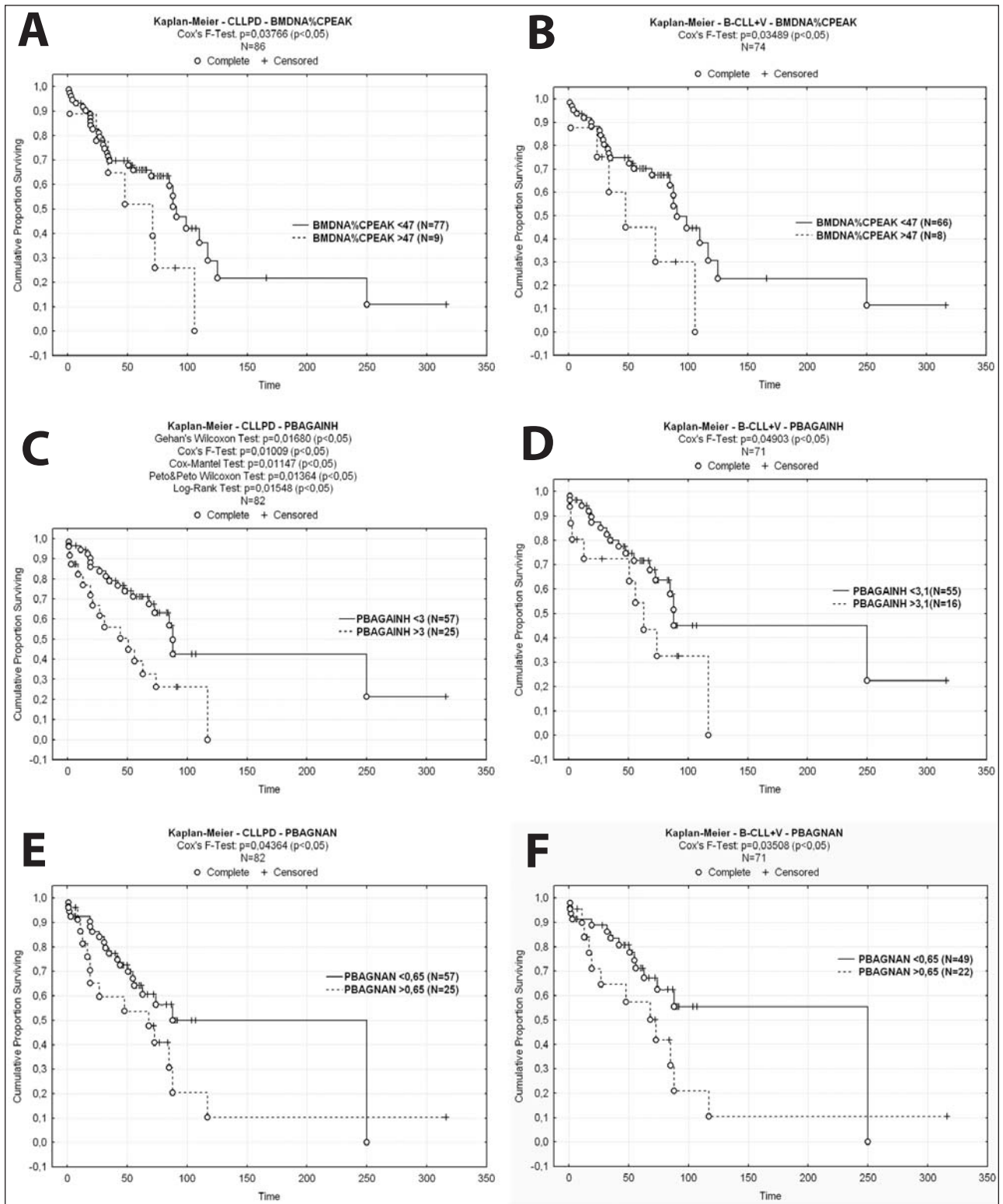


Figure 3 Patient survival curves in relation to the percentage of cells in the peak of DNA histogram of lymphatic cells in the bone marrow of patients with (A) CLLPD and (B) B-CLL+V; the area of inhomogeneous AgNOR of lymphatic cells in peripheral blood of patients with (C) CLLPD and (D) B-CLL+V; number of annular AgNOR of lymphatic cells in peripheral blood of patients with (E) CLLPD and (F) B-CLL+V.

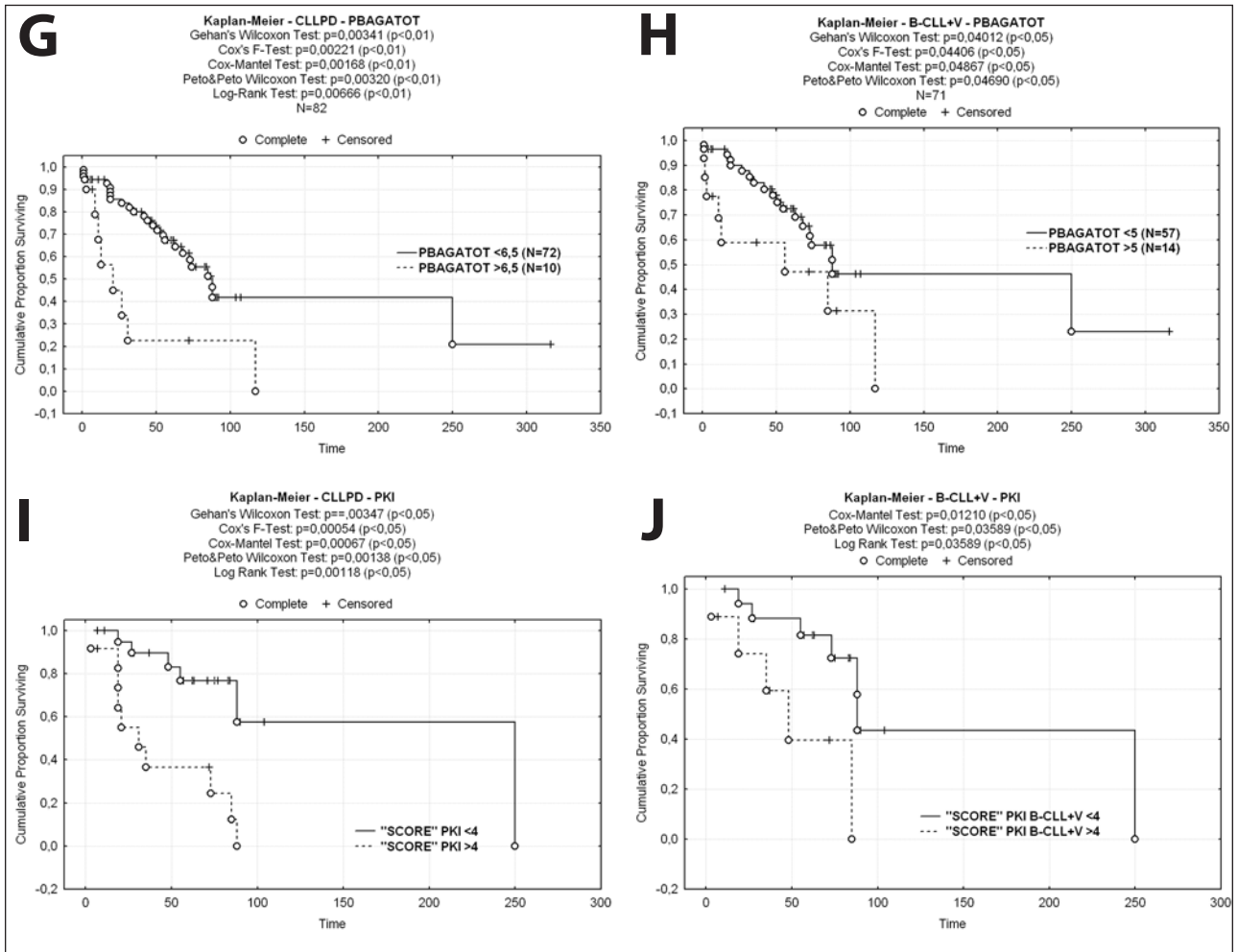


Figure 3 (cont'd) Total area of AgNOR of lymphatic cells in peripheral blood of patients with (G) CLLPD and (H) B-CLL+V; proliferative-kinetic index score for (I) CLLPD and (J) B-CLL+V.

of all AgNORs being $<6.4 \mu\text{m}^2$, as well as the total number of AgNORs in the bone marrow being <1.6 , and the proportion of the area of all AgNORs in relation to the nuclear surface being <0.07 . In peripheral blood, the number of homogeneous AgNORs was >0.3 ; the area was $>0.3 \mu\text{m}^2$ and the proportion of homogeneous AgNORs in relation to the nuclear surface was >0.002 in a more favorable prognosis. Furthermore, in the patients with a better prognosis, the area of inhomogeneous AgNORs was $<3 \mu\text{m}^2$ (Figure 3C), the maximum value of the area also was $<3 \mu\text{m}^2$ and the proportion in relation to the nuclear surface was <0.04 . They had the number of anular AgNORs that were <0.65 (Figure 3E), with the area $<2.3 \mu\text{m}^2$ and a maximum value of the area of anular AgNORs $<3 \mu\text{m}^2$. The number

of total AgNORs that was >1.37 , the area of total AgNORs that was $<6.5 \mu\text{m}^2$ (Figure 3G) and the proportion of the area of all AgNORs in relation to the nuclear surface that was <0.07 were associated with a more favorable outcome of the disease. In lymph nodes, the area of inhomogeneous AgNORs that was $>3.674 \mu\text{m}^2$ and the total area of all AgNORs in relation to the nuclear surface that was >0.065 was associated with a more adverse prognosis.

In the B-CLL+V subgroup, poorer survival was observed in patients in whom the proportion of the area of homogeneous AgNORs in relation to the nuclear surface in the bone marrow was >0.004 , the total number of AgNORs in the bone marrow was >1.65 , the total AgNOR area was $<4 \mu\text{m}^2$ and the proportion of the area of all AgNORs in relation to

the nuclear surface was <0.06 . In peripheral blood the number of homogeneous AgNORs being <0.3 and the area being $<0.3 \mu\text{m}^2$ were predictors of a poorer survival. The area of inhomogeneous AgNORs being $>3.1 \mu\text{m}^2$ (Figure 3D), the maximum value of the area also being $>3.1 \mu\text{m}^2$ and the proportion of inhomogeneous AgNORs in relation to the nuclear surface being >0.047 classified the patients as having a poorer prognosis. In contrast, the number of annular AgNORs being <0.65 (Figure 3F), the maximum area of the annular AgNORs being $<3 \mu\text{m}^2$, the area of all AgNORs being $<5 \mu\text{m}^2$ (Figure 3H) and the proportion of the area of all AgNORs in relation to the nuclear surface being <0.07 were indicative of a more favorable outcome.

Proliferative-Kinetic Index for CLLPD and B-CLL+V

Based on the statistically significant differences in survival among patients with CLLPD and B-CLL+V, the most representative ICM and AgNOR parameters were selected (Table II) in order to define the proliferative-kinetic index (PKI) as an independent prognostic parameter. A statistically significant, more favorable prognosis was associated with the patients with CLLPD and in the B-CLL+V subgroup when the PKI was <4 (Figure 3I and J).

Discussion

PKI, based on the score of the most representative DNA and AgNOR characteristics related to sur-

vival and analyzed in various tumor mass compartments, has proved to be a good prognostic parameter in low proliferative tumors—in this case, leukemic types of chronic lymphoproliferative disorders. In the total CLLPD population and in the B-CLL+V subgroup, a PKI <4 was indicative of a more favorable prognosis.

The clinical picture of CLLPD shows considerable heterogeneity; the median survival varies widely, ranging from 2 to 20 years for different stages of the disease. Such an unpredictable course motivated many clinical hematologists and oncologists for >30 years to search for novel parameters that will predict survival and progression of the disease.²³ In this paper, we show that innovative analytical methodology of DNA and AgNOR on cytologic smears can be successfully combined in a new, highly significant prognostic index.

There are few data in the literature on the measurement of the amount of DNA in lymphatic cells in CLL, because this is a neoplasm of low malignant potential with a diploid DI and low proliferative activity.²⁴ Studies of S-phase determination by flow cytometry have shown that this parameter can be associated with a poorer prognosis, but none of the findings of these studies have been tested in large studies by multivariate analysis.²⁵ A low percentage of cells in the S-phase has been found in the peripheral blood of patients with CLL (2 ± 2),²⁶ although there also have been studies with a larger

Table II Score of the Most Representative Variables of PKI for CLLPD and B-CLL+V

Parameters	CLLPD		B-CLL+V	
	Category	Point	Category	Point
Percentage of cells with lower contents of DNA than cells in peak of DNA histogram (BM)	$>20\%$	0	$>20\%$	0
	$\leq 20\%$	1	$\leq 20\%$	1
Percentage of cells with higher contents DNA than cells in peak of DNA histogram (PB)	$\leq 45\%$	0	$\leq 45\%$	0
	$>45\%$	1	$>45\%$	1
Percentage of cells in S-phase of DNA histogram (BM)	$>4.1\%$	0	$>4.1\%$	0
	$\leq 4.1\%$	1	$\leq 4.1\%$	1
Percentage of cells in peak of DNA histogram (BM)	28–47%	0	33–47%	0
	$\leq 28\%$	1	$\leq 33\%$	1
	$>47\%$	1	$>47\%$	1
Area of homogeneous AgNOR (PB)	$>0.3 \mu\text{m}^2$	0	$>0.3 \mu\text{m}^2$	0
	$\leq 0.3 \mu\text{m}^2$	1	$\leq 0.3 \mu\text{m}^2$	1
Area of inhomogeneous AgNOR (PB)	$\leq 3.0 \mu\text{m}^2$	0	$\leq 3.1 \mu\text{m}^2$	0
	$>3.0 \mu\text{m}^2$	1	$>3.1 \mu\text{m}^2$	1
No. of annular AgNOR (PB)	≤ 0.65	0	≤ 0.65	0
	>0.65	1	>0.65	1
Total area of AgNOR (PB)	$\leq 6.5 \mu\text{m}^2$	0	$>4 \mu\text{m}^2$	0
	$>6.5 \mu\text{m}^2$	1	$\leq 4 \mu\text{m}^2$	1

BM = bone marrow, PB = peripheral blood.

total number of cells in the S-phase that correlated with a shorter treatment-free period and a shorter total survival.²⁷ New parameters of the modified analysis of a diploid-type histogram have proved to be adequate for kinetic analysis by ICM. Generally, favorable prognostic parameters include a higher number of cells in the peak of a DNA histogram and a higher number of cells with lower contents of DNA than cells in the peak, and unfavorable prognostic parameters include a higher percentage of cells with higher DNA contents than cells in the peak, cells with higher DNA contents than 4N and higher number of cells in the S-phase of the cell cycle. An observation of interest in this study was the improved survival of patients who had a slightly higher proliferative activity in the bone marrow (<47% of lymphatic cells in the peak, but not <28.2%, and a higher percentage of cells in the S-phase of the cell cycle), which is most probably related to apoptosis. The previous thesis that only dividing cells undergo apoptosis, which is also the mechanism of therapeutic response, has been confirmed. Proapoptotic activity of therapeutics, which leads to inhibition of RNA synthesis or alteration of DNA repair, is not possible if cells are not dividing.²⁸ Low proliferative activity in the bone marrow results in an accumulation of long-lived, functionally inactive neoplastic B-lymphocytes arrested in the G0 phase of the cell cycle. Similar results have been described (although in a peripheral blood sample) by Vrhovac et al,²⁹ in which a high expression of p27* correlated considerably with the tumor doubling time and lymphocyte number and was associated with unfavorable total survival. This lends support to the theory that clonal excess of B-lymphocytes is due to the decrease in cell death caused by low proliferative activity rather than the increase in cell proliferation.³⁰

The structure of the AgNOR in most lymphatic cells in CLL is presented as 1 large dot. Moderately large to large cells of PLL, HCL or Richter syndrome demonstrate various AgNORs and complex configurations.³¹ Some researchers believe that the AgNOR structure is useless in differentiating CLL from PLL.³² When discussing CLL, Lorand-Metze and Metz³³ also describe AgNORs as compact or larger heterogeneous precipitates, so-called clusters within or dots outside the structure of nucleoli. Lymphocytes containing 1 AgNOR cluster correspond with the circulating proliferative fraction in patients with CLL. The percentage of cells with AgNOR aggregates is an additional independent

prognostic factor of the stable phase of the disease (i.e., treatment-free period).³⁴ Patients with a high percentage of AgNOR aggregates or high total tumor mass values³⁵ have a lower capacity of spontaneous apoptosis *in vitro*.³⁶ The high AgNOR proliferative index (the percentage of nuclei with ≥ 5 AgNORs per nucleus) is in correlation with the interleukin-2-receptor (IL-2R) values and reflects proliferative activity. The mean AgNOR count correlates more with ploidy. This implies the use of the simple AgNOR method as an alternative kinetic method for cases in which other techniques, such as flow cytometry, determination of Ki-67 index or IL-2R expression, are not feasible.³⁷ In our study, AgNORs that were inhomogeneous in number and size were poorer prognostic parameters than were annular AgNORs. Total area and number of AgNORs also play a role in survival. The best survival is observed in patients who have lymphatic cells with small, homogeneous AgNORs.

Differences in biologic characteristics of tumor cells in various tumor mass compartments (bone marrow, peripheral blood and lymph node) may be clinically valuable for evaluation of CLLPD or B-CLL+V aggressiveness and identification of subgroups with more unfavorable clinical courses. This has been clearly demonstrated by the PKI's highly significant prognostic power. It offers a new option that may be used as a criterion for the selection of various therapeutic protocols. Based on the results presented in this study, the novel analysis of the diploid histogram and the newly characterized types of AgNOR structures could also be extended to the analysis of the proliferation of other diploid tumors of low malignant potential (e.g., epithelial, mesenchymal, or originating from other cells). This would also give credence to the idea that within the same morphologic group and the same clinical presentation different subgroups can be differentiated in terms of clinical course and influence on survival.

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