Argyrophilic nucleolar organizer regions (AgNORs) in relation to p53 and bcl-2 protein expression in leukemia patients*

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The aim of this study was to assess the possible relationship between the silver stained nucleolar organizer regions (AgNOR) and immunocytochemically detected p53 and bcl-2 proteins in ALL, AML, B-CLL and CML patients (adults and children) at the initial presentation. AgNORs are loops of DNA, correlated with proliferative potential of cells. Alteration in p53 and bcl-2 proteins expression may characterize the malignant potential of leukemic cells. The patients were subdivided according to the p53 positivity and negativity. The frequency of p53-positive patients was relatively low in T-ALL (29%) and B-CLL (16%). B-ALL, AML and CML patients revealed higher frequency of p53 protein (46%, 47% and 88%, respectively). The overall frequency of positive cytoplasmic staining for bcl-2 protein was demonstrated in the majority of patients. No significant differences in the percentage of p53-positive cells among leukemia subtypes were seen. The proportion of bcl-2 protein positive cells did not differ significantly among various leukemia subtypes, except for significant differences between p53-positive and p53-negative peripheral blood ($p=0.0073$) and bone marrow ($p=0.0175$) cells of B-CLL patients. The samples from healthy subjects used as controls exhibited relatively low numbers of AgNOR dots in both, peripheral blood and bone marrow cells. Highly significant differences in AgNOR quantities between healthy donors and p53 protein positive peripheral blood as well as bone marrow cells of distinct leukemia subtypes (except for bone marrow cells in B-CLL patients, $p=0.1727$) were observed. Significant differences in AgNOR count between p53 protein positive and p53 protein negative samples of peripheral blood cells of B-ALL ($p=0.0099$) as well as B-CLL ($p=0.0117$) cases were found. No significant differences (except for B-CLL, $p=0.0558$) were encountered in bone marrow cells. P53 protein positivity or negativity did not influence the amount of AgNOR proteins in cells of our T-ALL and AML cases. Mutual comparing the number of AgNOR dots among different leukemias showed that for both peripheral blood and bone marrow cells the differences between ALL and AML ($p=0.0383$ and $p=0.0033$, respectively) as well as for peripheral blood of AML and CML ($p=0.0302$) were statistically significant. The bcl-2 protein positivity did not affect significantly the AgNOR distribution either in p53 protein positive or p53-negative cases of our leukemia patients. However, an association between the lowest AgNOR quantity and highest bcl-2 protein expression in p53-negative B-CLL patients was seen for both peripheral blood and bone marrow cells. The correlation between relatively high AgNOR numbers and relatively increased percentage of bcl-2 protein in the p53-positive cases of CML patients was found in some cases. Regarding the age and sex, the AgNOR distribution in p53-positive and p53-negative leukemia cases in children and adults showed neither relationship nor dependence. The WBC count differed evidently among distinct leukemia subtypes, with enormous heterogeneity in range as well.

Larger studies are needed in order to consolidate these preliminary results and characterize the possible prognostic value of AgNOR in association with p53 and bcl-2 proteins expression.

Key words: AgNOR, p53 protein, bcl-2 protein, acute leukemia, chronic lymphocytic leukemia, chronic myeloid leukemia and immunocytochemistry.

Silver-stained proteins associated with nucleolar organizer regions (AgNOR) are widely applied in tumor pathology to study the biological properties of neoplastic cells. A number of investigations documented their importance as diagnostic and prognostic tool [5, 18, 24]. Their quantity is associated with the nucleolar biosynthetic activity with respect to ribosomal RNA transcription [5]. The number of AgNOR proteins has been shown useful in distinguishing malignant from benign neoplasms. It was found out that

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malignant cells frequently present a greater AgNOR protein amount than corresponding non-malignant cells [5, 24]. The AgNOR content of tumor cells, irrespective of their origin, is closely related to cellular proliferative activity [21, 23, 24]. The AgNOR protein quantity was thus proposed as a marker of proliferative activity. AgNOR quantification represents a valuable parameter of cell kinetics [21]. In contrast to precisely documented diagnostic and prognostic utility of AgNOR enumeration in solid tumors, lack of detail information is available regarding the relevance of AgNOR proteins in leukemias [7, 8, 13, 17, 22].

The p53, a nuclear phosphoprotein, product of the tumor suppressor p53 gene, plays a critical role in regulation of cell proliferation [16]. Tumor suppressor activity of p53 (wild-type p53) is usually explained by its ability to prevent expansion of potentially malignant cells by either induction of apoptosis or arrest in G1 phase of the cell cycle. Loss of normal growth-inhibitor activity of p53 protein, as a consequence of mutation, is a key event in tumorigenesis [6]. Mutated version of the p53 protein tends to accumulate into nuclei of malignant cells due to its longer half-life, thus becoming immunohistochemically detectable. Close correlation between positive immunocytochemistry for p53 and the presence of p53 missense mutation was suggested by numerous studies [4, 14].

The frequency of p53 mutations detected by PCR-SSCP, in hematological malignancies is relatively low compared to other tumors, but when present, they are important determinants of disease progression and poor prognosis [11, 12]. Correlations between p53 mutations, altered apoptosis and drug resistance were found in hematological malignancies and leukemias [16].

Bcl-2 protein is known to belong to a family of apoptosis (programmed cell death) regulatory gene products with the function to block the apoptosis. High expression of bcl-2 is associated with poor response to chemotherapy [3]. The important interactions between p53 and bcl-2 have been found in different leukemia subtypes [9, 10, 19].

Evaluation of AgNOR quantitative distribution represents an important tool to gain information on the proliferative activity of malignant cells [5].

The purpose of the present study was to investigate possible relationship between the quantities of AgNORs and immunocytochemically detected p53 and bcl-2 proteins expression in cells of patients with acute and chronic leukemias at the time of initial diagnosis and to assess their proliferative activity.

Material and methods

Patients and leukemia samples. The study included a group of ninety-nine patients, both children and adults, with the newly diagnosed, previously untreated acute and chronic leukemias (ALL, AML, B-CLL, CML). Thirteen patients were diagnosed as B-lineage acute lymphoblastic leukemia (B-ALL), seven were T-lineage acute lymphoblastic leukemia (T-ALL), thirty-four were acute myeloid leukemia (AML), thirty-seven suffered from B cell chronic lymphocytic leukemia (B-CLL) and eight were chronic myeloid leukemia (CML). For detail see Table 1.

Within acute and chronic leukemia subtypes there was a wide variation in WBC counts. Moreover, the remarkable differences were found between peripheral blood (PB) and bone marrow (BM) cells. The patients with T-ALL and CML exhibited higher WBC count than patients with B-ALL, AML and B-CLL. The diagnosis of leukemia patients was based on morphologic and cytochemical criteria according to the standard FAB classification and on immunophenotyping. PB and BM cells were stained with May-Grünwald-Giemsa.

Immunophenotyping. PB and BM cells were isolated by a standard density gradient centrifugation at the time of initial diagnosis, before any treatment. The cell surface antigens were detected by an indirect immunofluorescence assay using a panel of monoclonal antibodies (MoAbs) to lymphoid (B and T), myeloid and non-lineage specific antigens. MoAbs were purchased from Immunotech a Beckman Coulter Company, (Marseille, France). Flow cytometric analysis was performed on both, FACStar (Becton-Dickinson, USA) and EPICS ALTRA Flow cytometer (Beckman Coulter International S.A., USA). Cases with more than 60% of mononuclear cells reactive with an appropriate antibody were considered positive. PB and BM cells of 15 healthy subjects were studied simultaneously.

Immunocytochemical detection of p53 and bcl-2 proteins. Immunocytochemical analysis was performed on air-dried smears of freshly isolated PB and BM cells. To estimate the p53 protein expression, p53 specific mouse antihuman immunoreagent clone DO-1 and Bp53.12 that recognize both the wild type and mutated p53 protein were used (MoAbs were kindly provided by Prof. J. Kovařík, Brno, Czech Republic). To detect bcl-2, we used a MoAb that recognizes the 26-kD Bcl-2 protein (clone 100, isotype IgG1 mouse, Immunotech a Beckman Coulter Company, Marseille, France). For evaluation of p53 and bcl-2 proteins, a sensitive Immunotech detection kit (Immunotech a Beckman Coulter Company, Marseille, France) based on peroxidase labeled streptavidin biotin reagents were utilized, described in detail previously [9]. The percentage of positive cells was calculated after examination of at least 500 cells by light microscopy with oil immersion (magnification x 100) per sample. The leukemia patients were considered p53 protein positive when at least 10 % of neoplastic cells showed nuclear staining for the DO-1 or Bp53.12 MoAbs. For bcl-2, less than 20% positive cells were used as the cut-off to define negative cases.

AgNOR *staining method and counting.* Air-dried PB and

Table 1. Patients' characteristics at the time of initial diagnosis

Variables		Total number of patients (n=99) B-ALL n=13	T-ALL n=7	AML n=34	B-CLL n=37	CML n=8
Age	≤17	10.2 ± 5.9(7)[a]	9.0 ± 7.0(3)	8.8 ± 3.9(7)		13 yrs(1)
	≥17	47.5 ± 15.9(6)	43.2 ± 12(4)	54.0 ± 16(27)	59.5 ± 10.5	39.7 ± 17.6(7)
Sex	M	6(46)[b]	4(57)	15(44)	23 (62)	4(50)
	F	7(54)	3(43)	19(56)	14(38)	4(50)
WBC($x10^9$/L)		37.4 ± 39.9[c] (5.3–120)[d]	153.4 ± 136.6 (22–330)	65.0 ± 81.4 (1.9–300)	55.2 ± 74.1 (1.7–320)	157.2 ± 211.5 (6.2–465)
		57.0 ± 85.3[e] (12.1–280)[d]	206.1 ± 108.8 (46.9–345)	64.0 ± 63.9 (1.8–255)	89.1 ± 109.8 (3.6–500)	176.0 ± 220.3 (20.5–470)
p53						
positive		6(46)	2(29)	16(47)	6(16)	7(88)
negative		7(54)	5(71)	18(53)	31(84)	1(12)
bcl-2		n=11	n=5	n=18	n=37	n=8
positive		7(64)	3(60)	15(83)	37(100)	6(75)
negative		4(36)	2(40)	3(17)		2(25)

[a]median ± S.D. (number of patients), [b]number of patients (%), [c]white blood cell count in peripheral blood ($x10^9$/L) (PB), [d](range), [e]white blood cell count in bone marrow (BM).

BM cell smears were fixed in cooled (4 °C) ethanol for 5 min and rinsed with deionized distilled water. The smears were stained with silver method, as described previously [7]. Briefly, one volume of 2% gelatin solution in 1% formic acid was rapidly mixed with two volumes of a 50% silver nitrate. The slides were immediately immersed in this solution and incubated for 20–30 min at room temperature in the dark. The smears were then washed with distilled water and dipped in 5% sodium thiosulphate to remove the background silver absorption. No counterstain was used. Ag-NOR quantity was assessed by direct examination under the light microscope. At least 300 cells of each sample were examined using an x 100 oil immersion lens. The mean numbers of AgNOR dots per cell nuclei were calculated.

Statistical analysis. Student's t-test for equal and unequal variance was used to analyze the statistical significance of the results. Values of $p<0.05$ were considered to indicate a statistically significant difference.

Results

Table 1 shows general characteristics for the whole series of 99 leukemia patients at initial presentation. The WBC count differed evidently among distinct leukemia subtypes, with enormous heterogeneity in range as well.

In the first set of examinations, the patients were subdivided according to the presence or absence of p53 protein expression. The overexpression of p53 nuclear immunopositivity was detected in 6 of 13 (46%) B-ALL patients, 7 of 13 (54%) were negative (the cases with less than 10% of p53-positive cells were considered to be p53 protein negative). In T-ALL, only 2 of 7 (29%) patients revealed p53-positivity, the rest 5 cases (71%) were p53-negative. The overall frequency of p53-positive AML cases was 16 of 34 (47%), whereas remaining 18 (53%) patients were negative. In the group of 37 B-CLL patients, 6 (16%) were p53-positive and 31 (84%) displayed negativity. The majority of CML patients (7 of 8; 88%) exhibited p53 positive cells.

Simultaneously, but not in all leukemia cases, the bcl-2 protein expression was evaluated. Positive cytoplasmic staining for bcl-2 protein was demonstrated in 7 of 11 (64%) cases of B-ALL, 4 (36%) were bcl-2-negative. Among the 5 T-ALL patients 3 (60%) displayed bcl-2-positivity, the rest 2 (40%) were without bcl-2 expression. In AML patients 15 of 18 (83%) were bcl-2-positive and 3 (17%) were shown to be negative. It was worth noting that all B-CLL (100%) cases exhibited bcl-2 protein positivity. Out of 8 CML patients, 6 (75%) were bcl-2-positive and 2 (25%) revealed negativity (Tab. 1).

The percentage of p53 protein positive cells remarkably varied in individual patients (from 10% to 76% for PB and from 10% to 88% for BM, respectively). However, the differences in the mean percentage of p53-positive cells among examined leukemia subtypes were not statistically significant (Fig. 1). Out of 37 B-CLL patients, BM cells were assessed in 16 of them.

Likewise, the proportion of bcl-2 protein positive cells, although heterogeneous in range (from 20% to 92% for PB

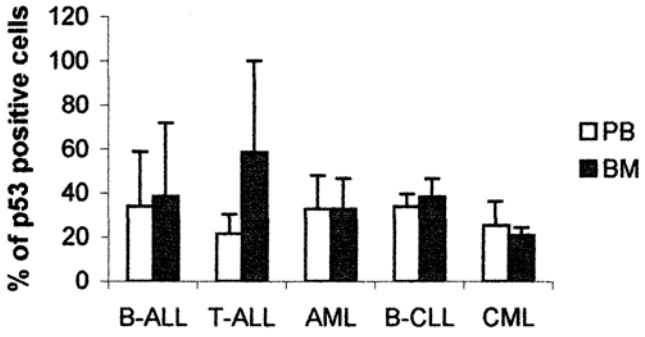

Figure 1. Percentage of p53-positive cells in peripheral blood (PB) and bone marrow (BM) cells in individual subtypes of leukemia.

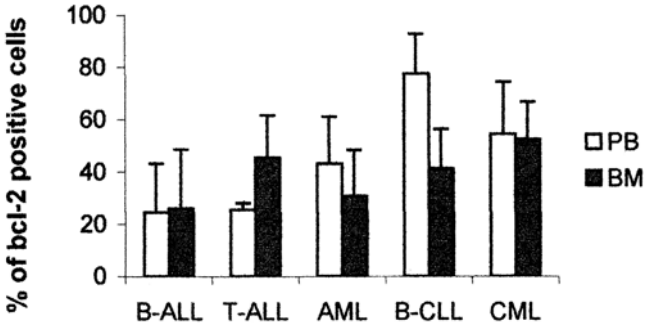

Figure 2. Percentage of bcl-2-positive cells in PB and BM cells in different leukemia phenotypes.

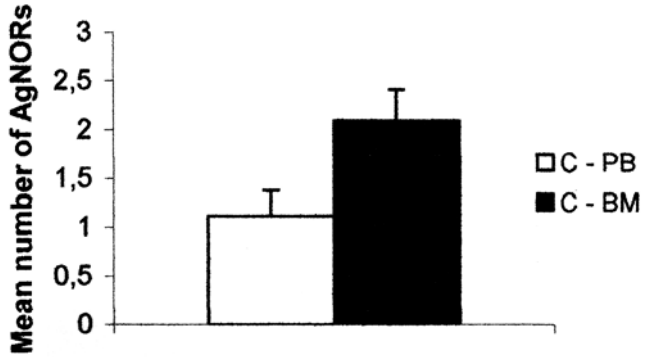

Figure 3. Mean number of AgNOR dots in PB and BM cells in healthy donors.

and from 20% to 74% for BM of individual patients, respectively) did not differ significantly among various leukemia subtypes, except for statistically significant differences between p53-positive and p53-negative peripheral blood (p=0.0073) and bone marrow (p=0.0175) cells of B-CLL patients (Fig. 2, Tab. 4).

To assess the effect of p53 protein presence or absence on the number of silver-stained nucleolar organizer region-related proteins (AgNORs), the quantitative analysis of the number of AgNOR dots per cell nucleus in both peripheral blood and bone marrow samples of different leukemia sub-

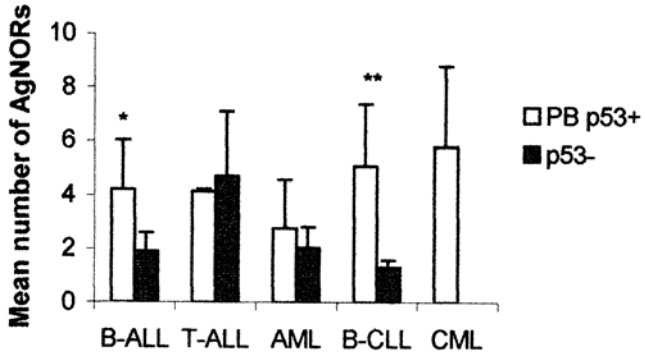

Figure 4. Mean number of AgNORs in PB of p53 protein positive and p53-negative cases of leukemia, *the differences are significant for B-ALL (p=0.0099) and **for B-CLL (p=0.0117).

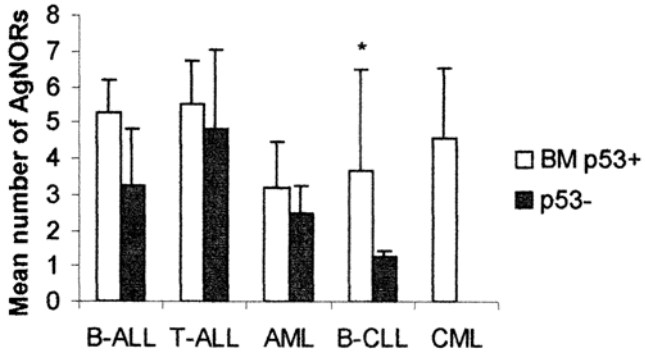

Figure 5. Mean number of AgNOR dots in BM of p53-positive and p53-negative cases of different leukemia subtypes, *the difference is significant for B-CLL (p=0.0558).

types as well as healthy donors was performed. AgNOR proteins were demonstrated as clearly visible black dots in the cell nucleus. Some of the dots had the tendency to form clusters or large aggregates. The percentage of cells exhibiting five or more AgNOR granules per nucleus ranged from 6% to 58%. Intranuclear silver deposits and their variability in numbers are shown in Figure 6.

The samples from healthy subjects used as controls exhibited relatively low numbers of AgNOR dots in both, peripheral blood and bone marrow cells (Fig. 3). However, highly significant differences in AgNOR quantities between healthy donors and p53 protein positive peripheral blood and bone marrow cells of distinct leukemia subtypes (except for bone marrow cells in B-CLL patients, p=0.1727) were observed (Tab. 2). It was worth noting a significant difference in AgNOR count between p53 protein positive and p53 protein negative samples of peripheral blood cells of B-ALL (p=0.0099) as well as B-CLL (p=0.0117) cases, but no significant differences (except for B-CLL, p=0.0558) were encountered in bone marrow cells. In addition, p53 protein positivity or negativity did not influence the amount of AgNOR proteins in cells of our T-ALL and AML cases (Fig. 4 and 5).

Table 2. Relationship between the AgNOR counts in p53 protein positive leukemia cases and in healthy subjects

	PB	p value	BM	p value
Control (n=15)	1.17±0.2(0.79–1.93)[a]		2.09±0.3(1.69–2.88)	
B-ALL (n=6)	4.21±1.8 (1.26–6.81)	0.0041	5.28±0.9 (4.23–5.96)	0.0243
T-ALL (n=2)	4.11±0.1 (4.05–4.18)	0.0084	5.50±1.7(4.27–6.72)	0.0503
AML (n=16)	2.76±1.8 (1.08–8.32)	0.0116	3.21±1.2 (1.19–5.29)	0.0561
B-CLL (n=6)	5.07±2.3 (3.03–9.10)	0.0047	3.67±2.8(1.24–7.59)	0.1727[b]
CML (n=6)	5.78±3.0 (2.30–9.26)	0.0129	4.57±1.9 (3.02–6.76)	0.0539

[a]mean number of AgNORs per nucleus ± S.D. (range), [b]no significant difference between healthy donors and p53-positive B-CLL patients in mean number of AgNORs for BM (p=0.1727).

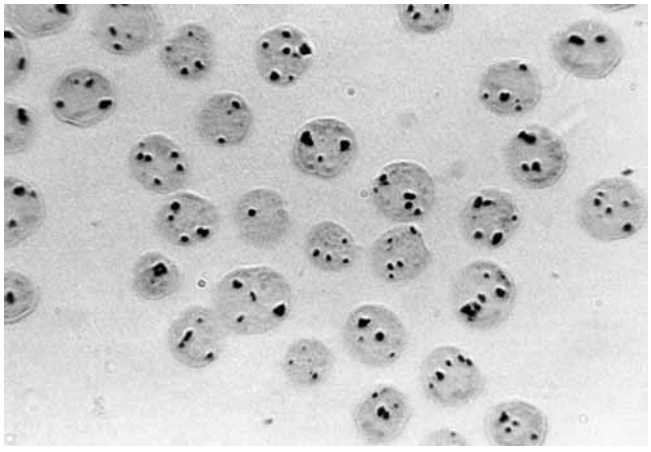

Figure 6. Silver stained AgNORs in peripheral blood cells of p53-positive T-ALL patient at diagnosis.

Table 3. Comparison of mean number of AgNORs among the distinct p53-positive leukemia phenotypes

	Mean number of AgNORs per nucleus ± S.D.			
Leukemia type	PB	p value	BM	p value
ALL (B and T)	4.16±1.1		5.39±0.9	
AML	2.76±1.8	0.0383[a]	3.21±1.2	0.0033[a]
ALL (B and T)	4.16±1.1		5.39±0.9	
B-CLL	5.07±2.3	0.2226	3.67±2.8	0.1621
AML	2.76±1.8		3.21±1.2	
CML	5.78±3.0	0.0302[b]	4.57±1.9	0.1766

[a]the difference is significant between ALL and AML for PB (p=0.0383) and BM cells (p=0.0033), [b]the difference is significant between AML and CML for PB cells (p=0.0302).

Comparison of the number of AgNOR dots among the different leukemias showed that the differences between p53-positive and p53-negative cases, for peripheral blood and bone marrow of ALL (B and T subtypes) and AML (p=0.0383 and p=0.0033, respectively) as well as for peripheral blood of AML and CML (p=0.0302) were statistically significant. No significant difference between p53-positive ALL and B-CLL in AgNOR count was seen (Tab. 3). In addition, the relatively high percentage of cells with the number of AgNOR dots more than 5 was the characteristic feature of many of the leukemia patients.

Table 4. Relationship between mean number of AgNORs and percentages of bcl-2 proteins in p53-positive and p53-negative leukemias

	p53-positive		p53-negative	
	AgNOR	% of bcl-2	AgNOR	% of bcl-2
B-ALL	4.21±1.8[a]	24.5±18.7	1.90±0.7	24.6±16.5
	5.28±0.9[b]	26.0±22.6	3.23±1.5	35.3±33.2
T-ALL	4.11±0.1	25.5±1.7	4.68±2.4	22.5±2.1
	5.50±1.7	29.3±19.4	4.83±2.2	36.0±4.2
AML	2.76±1.8	43.2±17.9	2.04±0.7	35.6±18.2
	3.21±1.2	30.7±17.7	2.40±0.8	29.2±10.3
B-CLL	5.07±2.3	41.6±16.4[c]	1.32±0.2	77.6±15.2[c]
	3.67±2.8	35.7±21.0[d]	1.28±0.1	80.0±8.2[d]
CML	5.78±3.0	54.5±20.0	One case only	
	4.57±1.9	52.6±14.3		

[a]mean number of AgNOR dots ±S.D. in peripheral blood cells (PB), [b]mean number of AgNOR dots ± S.D. in bone marrow cells (BM), [c]the difference is significant between percentage of bcl-2 in B-CLL p53-positive and p53-negative cases for PB (p=0.0073), [d]for BM (p=0.0175).

It was noted that the bcl-2 protein positivity only weakly affected the AgNOR distribution in p53 protein positive and p53-negative cases of our leukemia patients. Nevertheless, some exceptions were observed. An association between the lowest AgNOR quantity and highest bcl-2 protein expression in p53-negative B-CLL patients was seen for both peripheral blood and bone marrow cells. Moreover, the correlation between relatively high AgNOR numbers and relatively high percentage of bcl-2 protein in the p53-positive cases of CML patients was found (Tab. 4).

Concerning the distinct FAB groups of p53-positive cases of AML, the wide variation in the number of AgNORs was observed. The mean number of AgNOR counts was higher in FAB M5 (5.0±1.8) than in M1/M2 (4.2±0.9), M4 (4.0±1.6) or M0 (3.8±1.0). The two M3 cases were p53 protein negative. Moreover, among the eight, to our inves-

tigation available CML patients, only one, classified as stable chronic phase, revealed p53 protein negativity associated with relatively low numbers of AgNORs (2.4). The number of AgNORs in CML lymphoid blast crisis was higher (6.7) than in cells from CML in myeloid blast crisis (4.2). The highest number of AgNORs was seen in 3 CML cases (8.0 ± 0.3). The exact data were not shown because of the insufficient numbers of patients in individual AML and CML FAB subtypes.

Regarding the age and sex, the AgNOR distribution in p53 protein positive and p53-negative leukemia cases in children and adults showed neither relationship nor dependence. Despite the above-mentioned differences among distinct leukemia subtypes in WBC count, no remarkable effect of p53 protein presence or absence on WBC count was observed. Of note was only the association between high WBC count and p53-negativity in peripheral blood of AML patients ($p=0.0378$) (data not shown).

Discussion

In this study, the impact of p53 and bcl-2 protein expression on AgNOR amount in cells of acute and chronic leukemia subtypes at the presentation was assessed.

It is generally accepted that the overexpression of p53 protein (presumably corresponding to a mutated p53 in most cases) reflects the malignant character of cells [4, 11, 12, 16]. Moreover, mutant p53 can deregulate apoptosis resulting in cell proliferation and malignant transformation [20]. The balance between proliferation and apoptosis is important in controlling overall growth [28]. The essential role in the regulation of apoptosis has been attributed to bcl-2 (as programmed cell death inhibitor) as well as p53 genes and their protein products [16, 29]. The rate at which the tumor cells proliferate represents an important parameter for predicting the clinical outcome of patients with neoplastic disease [23]. If the rates of cell death exceed the proliferation rate, tumor regression will occur. Conversely, if proliferations exceed cell death, tumor progression will result [28]. The quantity of AgNORs can be used as a measurement of cellular proliferative activity [7, 21, 24].

Analysis of AgNORs originally regarded as a diagnostic tool is now considered as a prognostic parameter [18]. Moreover, AgNOR method can identify neoplastic clones with different proliferative activities and may stratify patients into different risk groups [5, 17, 22]. In hematological malignancies, according to our knowledge, no information is available on mutual interactions between p53, bcl-2 proteins and AgNORs.

In the present study, healthy subjects exhibited relatively low number of AgNOR proteins in both, peripheral blood and bone marrow cells in comparison with leukemia patients. This finding is supported by other reports [5, 22, 24]. Moreover, when patients were subdivided according to the presence or absence of p53 protein, the number of AgNORs significantly differed between healthy subjects and p53-positive cases as well as between p53-positive and p53-negative cases. It is supposed that the malignant cell proliferation induced by p53 alterations [16] may influence the AgNOR profile of different leukemia cell types.

The peripheral blood cells of B-ALL patients were significantly influenced by p53 protein presence at presentation. A significant difference in AgNOR amount between p53- positive and p53-negative B-ALL cases was characteristic. On the other hand, AgNOR quantification in our small group of T-ALL patients shows a tendency to be progressively increased, irrespective of p53 protein presence or absence. T-lineage ALL is distinct from B-lineage ALL not only phenotypically, but also biologically. T-ALLs are usually associated with high WBC count and unfavorable prognosis [27]. Moreover, a great deal of T-ALL, in opposite to B-ALL cases, revealed a high proportion of atypical/aberrant phenotypes [1]. In addition, the frequency of p53 mutations is low at diagnosis, but mutations are frequently associated with relapse in T-ALL [27].

In acute myeloid leukemia, the discrepancy between the low incidence of p53 mutation and the p53 protein overexpression has been reported [14]. Of our AML cases, 47% of patients displayed the nuclear p53 protein positivity. The literature data documented that immunostaining may be positive also in the absence of detectable p53 mutation and could correspond to overexpression of a non-mutated p53 [6, 14]. This fact appeared to be a likely explanation of the relatively low AgNOR quantities, regardless of p53 positivity or negativity in our AML cases. Our previous study on the relationship between AgNORs and some immunophenotypic markers in acute leukemia [8] suggested the low proliferative potential of AML. Myeloblasts of AML apparently seem to be less active with respect to the nucleolar biosynthetic activity and proliferation [25].

In this study, overall frequency of p53 protein positive B-CLL patients was 16% and the percentage of p53 immunoreactive cells remarkably varied between individual patients. AgNOR amount in p53-positive B-CLL cases was unexpectedly high, mainly in peripheral blood cells. Significant difference between p53-positive and negative cases in AgNOR counts was evident in both, peripheral blood and bone marrow cells. B-CLL is typically a low-grade neoplasia with a low proliferation activity and one or two AgNOR granules per cell nucleus [13]. However, Metze et al [13] have shown that B-CLL cells with clusters of AgNOR dots may represent the circulating proliferative fraction in CLL, presenting a good correlation to tumor mass and lymphocyte doubling time. Further, Cordone's et al [4] findings indicated that p53 protein expression in B-CLL is strongly associated with p53 gene mutation and p53 protein positive patients displayed a significantly shorter survival and poorer response to ther-

apy compared with p53-negative cases. The p53 mutations in B-CLL were associated with multiple cell cycle regulator disruptions that may facilitate the transformation to a more severe disease [16]. Thus, it is supposed that our p53 protein positive cases of B-CLL characterized by a numerous AgNOR dots might represent a subgroup of B-CLL with higher proliferation capacity.

Among the eight, to our study available CML cases, only one patient was p53 protein negative, others were p53 positive. The p53 positivity was associated with blast crisis, lymphoid or myeloid and high mean number of AgNORs. p53 protein negative patient was in chronic stable CML with low quantity of AgNOR. Accelerated phases and blast crisis of CML are usually correlated with alteration in p53 gene [11]. The proportion of cells in S-phase is a reliable indicator of proliferation. Strong association between a high AgNOR numbers and the percentage of cells in the S-phase (DNA synthesis phase) in blast crisis of CML patients was reported in our previous paper [7].

Comparative investigation of the p53-positive subtypes of leukemia according to the AgNORs showed significant differences between ALL and AML in both peripheral and bone marrow cells and between AML and CML in peripheral blood cells. No significant differences for AgNORs in relation to p53 expression between ALL and B-CLL were seen.

Regarding the bcl-2 protein, its expression was the characteristic feature of the majority of leukemia subtypes in our investigation. High expression of bcl-2 was reported to be associated with poor prognosis and unfavorable treatment outcome in AML [3]. Patients with AML exhibit chemotherapy resistance linked to cellular resistance to apoptosis [15]. On the contrary, Uckun et al [26] observed no association of bcl-2-positivity in newly diagnosed ALL with poor risk characteristics. An accumulation of malignant B-lymphocytes in B-CLL as the result of inhibited apoptosis has been studied [2].

Although our study is based on relatively small numbers of leukemia subtypes, results of investigation permit us to conclude that AgNOR enumeration in combination with p53 and bcl-2 protein detection in individual leukemic cells at diagnosis might provide a valuable information on their different proliferative activities. It is expected that long term follow up on the large numbers of patients would establish whether AgNOR in association with p53 and bcl-2 protein expression might stratified patients into different risk groups.

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