Diagnostic, predictive and prognostic verification of DNA flow cytometric measurements performed at diagnosis for non-Hodgkin's lymphoma adult patients

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More than ten years ago we made first attempts at valuating a prognostic power of flow cytometric DNA measurement results for patients with non-Hodgkin's lymphoma. In multivariate overall survival analysis, S-phase fraction (SPF) showed to be the only independent prognostic factor within the group of patients with low grade lymphomas. In this paper, we have tried to check our previous results in a greater group of patients with longer follow-up, within the specific types of B-cell and T/NK-cell lymphomas verified and classified according to criteria of the WHO 2008 classification. The study was performed on the material obtained from biopsies (85% of lymph nodes) of 484 NHL patients. Patients were diagnosed from 1991 to 2007. The medium follow-up time for living patients was 69 months (range: 25-202 months). All specimens were verified histologically and immunohistochemically. Ploidy and SPF were determined by flow cytometry on fresh tissue obtained during the diagnostic procedure. The diagnostic importance of ploidy and SPF has been confirmed. Ploidy had no predictive or prognostic impact in any of the NHL types, whereas SPF was found to be an independent predictive or prognostic factor in B-CLL/SLL, DLBCL and ALCL.

Key words: non-Hodgkin's lymphoma, flow cytometry, S-phase fraction, ploidy.

Introduction

More than ten years ago we made first attempts at valuating a prognostic power of flow cytometry DNA measurement results for patients with non-Hodgkin's lymphoma (NHL) [1]. Our group of patients was rather small (111 patients) and follow-up was short (median 25 months). The Kiel and Working Formulation classifications were used. Nevertheless, in multivariate overall survival analysis, S-phase fraction (SPF) showed to be the only independent prognostic factor within the group of patients with low-grade lymphomas. Since that time a significant progress in knowledge about NHL has been achieved, what was the impulse for developing a new REAL/WHO classification.

In this paper, we have tried to check our previous results in a greater group of patients with longer follow-up, within the specific types of B-cell and T/NK-cell lymphomas verified and classified according to criteria of the WHO 2008 classification [2].

The prognostic relevance of cell kinetics in malignant NHL has been studied for several decades [3-8], and many reports have confirmed the prognostic significance of SPF [5, 6]. Unfortunately, most studies were performed on NHL groups divided into low and high grade of malignancy (Kiel classification) or low, intermediate and high grade (Working Formulation).

Material and methods

Patients

Between January 1991 and December 2007, flow cytometry DNA measurements were performed for 573 patients examined in the Centre of Oncology in Kraków, Poland, and recorded as NHL patients. Four hundred and ninety-nine of them could be verified and reclassified according to the WHO 2008 classification with one exception: for this study, patients with DLBCL were considered as a uniform group.

In 484 cases, the diagnosis of NHL was confirmed, in 9 cases – reactive lymphocytes were found, in 5 cases – Hodgkin's disease was recognized and in 1 case – a cancer metastasis was present. In each case, the diagnosis of NHL was confirmed by histological and necessary additional immunohistochemical staining.

Among 484 patients with NHL, 335 patients were previously untreated. The median age of untreated patients at diagnosis was 64 years (range: 17-92 years) and 161 (48%) of them were males. Staging was performed according to Ann Arbor classification system. All patients received chemotherapy and/or irradiation (either combined or as the sole treatment), mostly according to standard protocols.

At the end of the observation, in October 2010, 110 patients were dead; 104 of progressing lymphoma and 6 of other causes. One hundred and thirty-seven patients are still alive: 74 without evidence of disease, 63 with lymphoma. The medium follow-up time for living patients was 69 months (25-202 months). Eighty-eight patients were lost during follow-up (diagnosed in the Oncology Center and cured in other hospitals).

Histology and immunochemistry

Imprints on the object glasses were prepared from the fresh tumor specimens supplied directly after surgery and then fixed in 10% buffered formaldehyde and/or 95% ethanol. After staining with hematoxylin/eosin (HE), they were cytologically evaluated. Fragments of fresh tumor tissue were sampled for flow cytometry and also routinely fixed and processed for histological examination. The microtome sections were stained with HE and according to PAS, Giemsa and Gomori procedures. Whenever it was necessary, additional immunohistochemical stainings were performed. Lymphomas were reclassified according to the WHO 2008 classification.

Flow cytometry

DNA analysis was performed on the suspensions of the cell nuclei from the fresh tissue specimens. After mincing with scissors, the tissue was disaggregated mechanically. Then, aliquots of 1-2 $\times$ 10^6 cells were incubated with the staining solution (PI – Calbiochem 50 μg/ml, NP-40 and RNA-se A – Sigma 1 mg/ml). For each DNA histogram, about 10 000-20 000 particles were analyzed. The median coefficient of variation (CV) of diploid peaks was 3.1% (range 1.4-9.5%). The DNA histograms were classified according to principles adopted at DNA Cytometry Consensus Conference 1992 [9]. In 1990-1996, measurements were performed on FACScan (with DDM module), further on FACSCalibur flow cytometer (Becton-Dickinson, argon laser 15 mW, 488 nm). The analyses were performed using ModFit.

Statistical analysis

Significance of differences between mean values was tested by Student's t-test. Frequency was compared by the χ^2 test. Correlations were checked by Spearman R-test. Disease-free survival (DFS) was calculated from the date of complete response (CR) to treatment, to the first recurrence. Patients with another type of response to treatment (partial response, stabilization or progression) did not have DFS. Overall survival (OS) was calculated from the date of diagnosis until death or date last known alive. The DFS and OS were assessed using the Kaplan-Meier method and compared between risk groups, applying the log-rank test. Prognostic parameters were estimated using Cox multivariate regression model. Cut-off points for the SPF were the median value for a particular group of NHL. Values of $p \leq 0.05$ were considered statistically significant.

Results

DNA content, SPF and histology

Ploidy and SPF could be estimated for all 484 NHL cases. Results are summarized in Tables I and II. Aneuploid cases were found in almost all types of B- and T-cell lymphomas, but with a different frequency. The most aneuploid rate in B-cell lymphoma was found in diffuse large B-cell lymphoma (DLBCL; 34.4%), mantle cell lymphoma (MCL; 29.8%) and follicular lymphoma (FL; 23.3%), whereas in the B-cell chronic lymphocytic leukemia/small lymphocytic leukemia (B-CLL/SLL) group, only one patient had aneuploid cell line (1.4%). It is worth noting differences in aneuploid DI compartments: in MCL, all aneuploid cases had near-tetraploid pattern, whereas in FL, aneuploidy was rather within the near-diploid region. In T/NK-cell lymphomas, the highest frequency of aneuploidy was found in anaplastic large cell lymphoma (ALCL; 63.6%) and lymphoblastic leukemia/lymphoma (T-LL; 33.3%).

Median level of SPF seems to be specific of each type of lymphoma. The two most characteristic are B-CLL/SLL with SPF $<$ 1% and Burkitt lymphoma (BL) with SPF near 50% of cells. In indolent types of NHL

Table I. B-cell neoplasms

	No. of patients	No. of previously untreated patients	No. of aneuploid[1] cases	Type of aneuploidy[2], no. of cases			% of SPF, median (range)
				ND	NT	Others	
B-lymphoblastic leukemia/lymphoma	9	8	2	2			20.7 (1.8-51.9)
Chronic lymphocytic leukemia/ small lymphocytic lymphoma	74	56	2	2			0.5 (0.1-4.2)
B-cell prolymphocytic leukemia	1	1	0				1.3
Lymphoplasmacytic lymphoma	2	1	0				4.4 (0.5-8.2)
Plasma cell myeloma	2	1	1	1			0.6 (0.1-1.1)
Marginal zone lymphoma	16	8	2	2			1.0 (0.2-10.0)
Follicular lymphoma grade 1	28	19	4	4			0.9 (0.2-3.1)
Follicular lymphoma grade 2	28	17	7	5	1	1	2.9 (0.4-10.3)
Follicular lymphoma grade 3	17	8	6	6			6.4 (1.3-16.8)
Mantle cell lymphoma	47	33	14		14		4.2 (0.3-46.0)
Diffuse large B-cell lymphoma	189	133	65	40	15	10	12.6 (0.3-44.5)
Burkitt lymphoma	4	4	1	1			49.35 (25.0-56.7)
B-cell lymphoma, unclassifiable	13	8	2	1	1		3.0 (0.2-31.9)
Methotrexate-associated lymphoproliferative disorder	4	2	0				6.5 (3.3-15.6)
All	434	299	106	64	31	11	5.4 (0.1-56.7)

[1]DI ≠ 1.00, [2]ND (near-diploid): 1.00 > DI ≤ 1.35, NT (near-tetraploid): DI > 1.8, Others: DI < 1.00 or 1.35 > DI ≤ 1.8

(FL1-2, marginal zone lymphoma – MZL), SPF is mainly low, whereas in lymphoblastic lymphoma B- and T-cells, DLBCL and ALCL, most cases have SPF > 10%. MCL, FL3 and angioimmunoblastic lymphoma (AILT) constitute an intermediate group.

DNA content, SPF and clinical data

Considered for establishing correlations between the studied factors and clinics following clinical parameters were included: sex, age (< 60 years vs. > 60 years), clinical stage (I-II vs. III-IV), performance status

Table II. T/NK-cell neoplasms

	No. of patients	No. of previously untreated patients	No. of aneuploid[1] cases	Type of aneuploidy[2], no. of cases			% of SPF, median (range)
				ND	NT	Others	
T-lymphoblastic leukemia/lymphoma	8	6	2	1	1		13.2 (4.6-24.3)
Aggressive NK cell leukemia	1	0	0				15.2
Enteropathy-associated T-cell lymphoma	1	0	0				45.6
Subcutaneous panniculitis-like T-cell lymphoma	1	1	0				6.3
Peripheral T-cell lymphoma	19	11	2	1		1	3.7 (0.6-17.8)
Angioimmunoblastic T-cell lymphoma	9	8	0				6.7 (2.4-12.7)
Anaplastic large cell lymphoma	11	10	7	1	5	1	14.0 (4.6-51.7)
All	50	36	11	3	6	2	7.4 (0.6-51.7)

[1]DI ≠ 1.00, [2]ND (near-diploid): 1.00 > DI ≤ 1.35, NT (near-tetraploid): DI > 1.8, Others: DI < 1.00 or 1.35 > DI ≤ 1.8

(0-2 vs. 3-4), symptoms (absent vs. present), LDH level (normal vs. increased), blood lymphocyte count (< 12 000 vs. > 12 000), response to therapy (CR vs. other), the end of disease-free survival (DFS) and overall survival (OS).

Analyses were done only for previously untreated patients. Multivariate and survival analyses were performed for patients with complete or higher than 24 months' follow-up.

B-cell lymphomas (299 patients)

B-lymphoblastic leukemia/lymphoma (B-LL)

Eight patients, 5 males and 3 females, with the median age of 49 years (range: 17-74 years). Four of them presented in III-IV clinical stage, four had a higher level of LDH (> 2×). Three patients died within the median 2 months (range: 1-5 months), 3 patients were alive without signs of disease for the median of 57 months (range: 27-111 months), 1 patient died of another cause after 38 months and one was lost during follow-up. Four of them achieved complete response.

Aneuploidy and SPF did not correlate with any clinical parameter and had no influence on response to therapy and on survival.

Chronic lymphocytic leukemia/small lymphocytic lymphoma (B-CLL/SLL)

Fifty-six patients, 31 males and 25 females with the median age of 66 years (range: 37-86 years). Fourty-four of them presented in III-IV clinical stage, 9 had a higher level of LDH. Ten patients died within the median of 23 months (range: 2-84 months), 30 patients were alive: 3 without (median: 101 months; range: 39-113 months) and 27 with (median: 58 months; range: 28-138 months) signs of disease, 16 patients were lost during follow-up.

Aneuploidy was demonstrated only in one patient and had no influence on survival. The SPF did not correlate with any clinical parameter. However, SPF higher than 0.5% proved to be the only parameter influencing overall survival in multivariate analysis (Table III, Fig. 1).

Follicular lymphoma (FL)

Fourty-four patients (19 FL1, 17 FL2, 8 FL3), 11 males and 33 females with the median age of 58 years (range: 29-81 years). Eighteen of them presented in III-IV clinical stage, 3 had a higher level of LDH. Six patients died within the median of 35 months (range: 11-100 months), 26 patients were alive: 14 without (median: 90 months; range: 34-176 months) and 12 with (median: 61 months; range: 38-162 months) signs of disease, 12 patients were lost during follow-up.

Aneuploidy did not correlate with any clinical parameter and had no influence on response to therapy and survival. Patients with lower SPF had a tendency (but not significant) to longer overall survival.

Mantle cell lymphoma (MCL)

Thirty-three patients, 21 males and 12 females with the median age of 70 years (range: 32-86 years). Twenty-six of them presented in III-IV clinical stage, 6 had a higher level of LDH. Thirteen patients died within the median of 10 months (range: 3-58 months), 8 patients were alive: 2 without (median: 33 months; range: 25-41 months) and 6 with (median: 40 months; range: 26-166 months) signs of disease, 12 patients were lost during follow-up.

Aneuploidy was correlated only with performance status ($p = 0.0041$, $r = 0.5000$). Among patients with the diploid pattern, only 2/22 (9%) had PS > 2, whereas within the aneuploid group, PS > 2 was found in 6/11 (55%) patients. The SPF did not correlate with any clinical parameter.

Aneuploidy and SPF had no influence on response to therapy and survival.

Diffuse large B-cell lymphoma (DLBCL)

One hundred and thirty-three patients, 60 males and 73 females with the median age of 64 years (range: 19-92 years). Sixty-five of them presented in III-IV clinical stage, 52 had a higher level of LDH. Fifty-five patients died within the median of 9 months (range: 1-61 months), 45 patients were alive: 37 without (median: 76 months; range: 25-202 months) and 8 with (median: 47 months; range: 26-113 months) signs of disease, 4 patients died of another cause (median: 79 months; range: 40-103 months) and 29 were lost during follow-up.

Table III. B-CLL/SLL: multivariate overall survival analysis

	RELATIVE RISK	VALUE OF P
SPF > 0.5%	5.29	0.0356

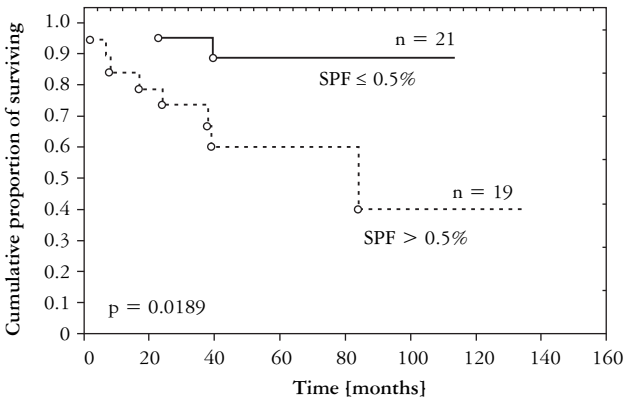

Fig. 1. B-CLL/SLL: relation of SPF value to overall survival of patients

Table IV. DLBCL: patients who achieved CR and patients with recurrence after CR with respect to SPF

	SPF ≤ 12.6%, NO. OF PATIENTS	SPF > 12.6%, NO. OF PATIENTS	VALUE OF P
Response to treatment:			
CR	26 (49%)	32 (65%)	0.09782
no CR	27 (51%)	17 (35%)	
Recurrence after CR:			
absent	12 (60%)	25 (89%)	0.02197
present	8 (40%)	3 (11%)	

Table V. DLBCL: multivariate analysis

PARAMETER	RELATIVE RISK	VALUE OF P
Disease-free survival		
Age > 60 years	2.05	0.0088
SPF > 12.6%	0.51	0.0109
Clinical grade III-IV	1.92	0.0151
Sex (male)	1.71	0.0361
Overall survival		
Age > 60 years	2.26	0.0054
SPF > 12.6%	0.59	0.0616
Clinical grade III-IV	2.18	0.0081

Table VI. T-LL: difference of median SPF between patients who achieved CR and other response to treatment

RESPONSE TO TREATMENT	NO	% OF SPF, MEDIAN (RANGE)	VALUE OF P
CR	4	15.7 (9.6-23.9)	0.0715
No CR	2	5.2 (4.6-5.8)	

Aneuploidy did not correlate with any clinical parameter and had no influence on response to therapy and survival. The SPF correlated with response to treatment (not statistically significant) and recurrences after complete response (Table IV). In multivariate analysis, SPF has occurred to be one of the independent factors together with age, clinical stage and sex, influencing disease-free survival (Table V), whereas for overall survival, SPF did not achieve statistical significance (Table V). Addition of SPF to IPI (International Prognostic Index) discriminated survival of patients with IPI 1-3, but not patients with IPI 4.

Marginal zone lymphoma (MZL)

Eight patients, 3 males and 5 females with the median age of 64 years (range: 57-70 years). Four of them presented in III-IV clinical stage, 1 had a higher level of LDH. Two patients died within the median of 49 months (range: 40-57 months), 6 patients were alive: 2 without (median 71 months; range: 43-99 months) and 4 with (median: 64 months; range: 58-144 months) signs of disease.

No case of aneuploidy was found in this type of neoplasm. The SPF did not correlate with any clinical parameter and had no influence on response to therapy and survival.

T-cell lymphomas (36 patients)

T lymphoblastic leukemia/lymphoma (T-LL)

Six patients, 3 males and 3 females with the median age of 23 years (range: 17-72 years). Six of them presented in III-IV clinical grade, 4 had a higher level of LDH. One patient died within 32 months, 4 patients were alive: 3 without (median: 155 months; range: 69-177 months) and 1 with (121 months) signs of disease, one patient was lost during follow-up.

One case of aneuploidy was found in this type of neoplasm. The SPF did not correlate with any of clinical parameters. The SPF had an influence (not statistically significant) on response to therapy (Table VI), but not on survival.

Peripheral T-cell lymphoma (PTCL)

Eleven patients, 5 males and 6 females with the median age of 55 years (range: 45-78 years). Eight of them presented in III-IV clinical grade, 2 had a higher level of LDH. One patient died within 1 month, 4 patients were alive: 1 without (43 months) and 3 with (median: 43 months; range: 25-75 months) signs of disease, 6 patients were lost during follow-up.

Ploidy and SPF did not demonstrate any correlation with clinical parameters and had no influence on response to therapy and survival.

Angioimmunoblastic T-cell lymphoma (AILT)

Eight patients, 7 males and 1 female with the median age of 62 years (range: 32-73 years). Eight of them presented in III-IV clinical grade, 2 had a higher level of LDH. Four patients died within the median 3 months (range: 1-12 months), one patient had been alive for 95 months without signs of disease and 3 patients were lost during follow-up.

Aneuploidy was absent in this group. The SPF demonstrated a strong correlation with age of patients ($p = 0.0003$, $r = 0.8729$); median SPF for 4 younger patients was 3.7% (range: 2.4-3.9%), whereas for 4 older patients, the median SPF was 8.0% (range: 6.7-8.9%). The SPF had an influence (but not statistically significant) on response to therapy ($p = 0.1161$, $r = 0.7071$) and overall survival ($p = 0.0259$, $r = -0.7684$). Patients with SPF lower than 5.3% had

the median of 54 months overall survival (range: 12-95 months) in comparison with the median of 1 month (range: 1-5 months) for patients with higher SPF.

Anaplastic large cell lymphoma (ALCL)

Ten patients, 5 males and 5 females with the median age of 47 years (range: 20-78 years). Three of them presented in III-IV clinical grade, 4 had a higher level of LDH. Three patients died within the median of 4 months (range: 1-6 months), 3 patients were alive for the median of 121 months (range: 74-134 months) without signs of disease, one patient died of another cause after 123 months and 3 patients were lost during follow-up.

Seven patients (70%) had an aneuploid pattern of DNA histogram. Aneuploidy correlated only with sex of patients (more frequently among females; $p = 0.0400$, $r = 0.6547$). The SPF correlated reversely with the LDH level ($p = 0.0472$, $r = -0.7500$) and had an influence (not statistically significant) on response to treatment (Table VII).

Additionally, patients with lower SPF (median: 13.4%, range: 13.2-13.5%) had a statistically significant ($p = 0.0054$) shorter overall survival (median: 3.5 months, range: 1.0-8.0 months) than those with higher SPF (median SPF: 29.1%, range: 15.2-51.7%; median overall survival: 122 months, range: 74-134 months).

Some types of lymphomas were overlooked due to an overly low number of patients for analysis.

Table VII. ALCL: differences of median SPF with respect to the LDH level and response to treatment

	No	% of SPF, median (range)	Value of p
LDH:			
normal	3	22.6 (15.2-35.5)	0.0472
higher	4	13.4 (11.4-51.7)	
Response to treatment:			
CR	5	22.6 (13.5-51.7)	0.0624
no CR	2	11.6 (9.9-13.2)	

Discussion

Ploidy

In comparison to other types of tumors (for example, breast cancer), aneuploidy in NHL is detected rather rarely. In this study, < 25% cases demonstrated the presence of aneuploid cell line. The distribution of DNA indices was generally bimodal: near-diploid and near- or hyper-tetraploid. Only 6/117 patients with aneuploid cell line had DI value of 1.3-1.7. A similar observation was published by Joensuu *et al.* [5]. In recent studies, aneuploidy was connected with defined types of lymphoma. A more precise examination of aneuploidy by DNA index (DI) could have a diagnostic importance for small lymphocyte lymphomas. As in follicular lymphoma, aneuploidy has mainly a near-diploid pattern, in mantle cell lymphoma, aneuploidy is, in general, near-tetraploid. Similar observations were published by others [10]. Ploidy did not have any predictive or prognostic value.

S-phase fraction

B-cell lymphomas

Percentage of cells in S-phase in B-cell lymphomas is characteristic of specific types of NHL and, for this reason, is a valuable complementary parameter for establishing a precise diagnosis. These results are in concordance with those obtained by others [10, 11].

The B-CLL/SLL is an example of neoplasm in which the number of cells increases not as a result of proliferation but accumulation, what reflects the minimal SPF value. From the group of indolent B-cell lymphomas, only B-CLL/SLL patients with a higher SPF level had a significantly shorter overall survival and SPF is the sole factor in multivariate analysis. A similar tendency was observed in patients with follicular lymphoma, but in a small number of previously untreated patients, it was not possible to check this factor in each histological grade of this disease type.

Among the more aggressive types of lymphoma, where the proliferation rate is higher and complete response is more probable to be achieved, the SPF level was important for DLBCL patients. Unexpectedly, patients with a higher proliferation rate achieved CR more frequently, rarely developed recurrence after CR and had longer disease-free and overall survival. These results are in contradiction to the most obtained by others, where the higher proliferation rate is almost always unprofitable [12]. However, conclusions similar to those presented in our study were published by Hasselblom *et al.* [13]. The study comprised a group of 199 patients with DLBCL and a low Ki-67 level (lower proliferation rate) was associated with worse prognosis, independent of clinical risk factors.

T-cell lymphomas

The SPF has an influence on response to therapy and survival in patients with AILT, but in this group of neoplasms, SPF strongly correlated with the age of patients and, as a result, the survival curves are identical. So, predictive and prognostic information could be obtained without measurement of SPF.

Among lymphoblastic lymphoma and ALCL, patients with a higher SPF presented a tendency to achieve complete response more frequently than those with

a lower SPF, but only ALCL patients with a higher SPF had a statistically significant longer overall survival.

In this study, both ploidy and SPF were the valuable diagnostic parameters. In some types of lymphoma, SPF confirmed its predictive and prognostic validity.

In general, in indolent lymphomas, a lower SPF value is connected with a longer overall survival, whereas in more aggressive lymphomas, patients with a higher SPF have greater probability to achieve complete response and a longer disease-free and overall survival.

The general treatment for the second group of patients is chemotherapy and for this type of treatment, the main target is proliferating fraction of cells. Thus, a higher level of SPF could be beneficial for patients with aggressive lymphomas.

In conclusion, flow cytometry DNA measurement should be a part of the routine work-up of the newly diagnosed NHL patients. Our findings, related to the predictive and prognostic role of SPF value in more aggressive lymphomas, should be confirmed in other studies.

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