Comparison of CD38, ZAP-70 and P53 markers on Disease Progression and Survival in B- Cell Chronic Lymphocytic Leukemia

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Abstract— *Chronic lymphocytic leukemia (CLL) is considered the most common B-cell leukemia. Its clinical course is highly variable and depends on clinical, biological and genetic features of leukemic B-cells. Expression of ZAP-70, CD38 and mutated p53 was assessed by flow cytometry methods in 175 B-CLL patients and estimated their impact on disease progression rate (DPR) and overall survival (OS). Fifty-one patients (29%) were ZAP-70(+), 81 (46%) were CD38(+), 24 (13.7%) were p53(+). OS was significantly shorter in CD38(+) patients (94.8 vs. 120 months, p<0.001). The similar tendency was found in ZAP-70(+) cases (88 vs.114 months, p<0.01) and in p53(+) patients (88.5 vs. 111.5 months, p=0.48). CD38, ZAP-70 and p53 positive patients have had 4-fold increased mortality rate then the patients with negative markers and this rate was significantly higher after the 6th year of the disease beginning. At multivariable analysis, combined CD38/ZAP-70/p53 status confirmed its independent prognostic role. Double positive CD38/ZAP-70, CD38/p53 and ZAP-70/p53 expressions showed over 19-fold increased DPR above the negative CLL cases (p<0.001). They were classified in a high risk CLL group. The single expressions of these markers were connected with lower DPR and were distributed in middle risk group. CD38, ZAP-70 and p53 negative patients were with the lowest DPR and were classified in the CLL group with good prognosis. ZAP-70 and CD38 expressions appeared to be more predictive than p53 expression and more relevant in defining the cases of B-CLL with higher disease progression rate.*

Keywords: Chronic Lymphocytic Leukemia (CLL), CD38, ZAP-70, p53 expression, survival study.

I. INTRODUCTION

Chronic lymphocytic leukemia (CLL) is considered the most common primary B-cell leukemia. Its clinical course is highly variable and a number of clinical and biological features have been used to separate CLL patients into subgroups with different prognosis and requirement of different therapeutic strategies. Leukemic B-cells may have several characteristics that are related with relatively aggressive disease. These include clinical stage, lymphocyte count and lymphocyte doubling time/ bone marrow infiltration, elevated lactate dehydrogenase (LDH), β2-microglobulin or thymidine kinase, genomic aberrations, gene mutations/deletions (p53, ATM), unmutated variable segments of immunoglobulin heavy chain genes (IgVH), or surrogate markers CD38 and ZAP-70.[1-5]

CD38 (also referred to as T10 antigen) expression is not limited only to T- cells but is widely expressed on different hematopoietic and non-hematopoietic cells.[6] It was found a strong correlation between the IgVH gene mutation status, CD38 surface expression of the respective B-CLL clone and disease

outcome.[1] Expression of CD38 on CLL cells has been shown to be increased in more proliferative clones.[7,8] A cutoff value for positivity in CD38 has been a source of discussion. Traditionally, surface expression of CD38 in > 30% of B-CLL cells was thought to be associated with more progressive disease.[4,9]

ZAP-70, a tyrosine kinase, is associated with T-cell development and differentiation. There is no ZAP-70 expression on normal B- lymphocytes, but it was detected on malignant B-CLL cells by flow cytometry.[10] It was suggested that the expression of ZAP-70 could serve as a prognostic factor in B-CLL and its expression would be stable during the course of the disease.[10] The significance of ZAP-70 as a surrogate marker for IgVH mutation was reported to help identify patients with a more aggressive clinical course.[10-12] A cutoff of 20%, ZAP-70 positivity clearly separated CLL patients into two groups; those with < 20% ZAP-70 had increased survival time and decreased chance of disease progression.[3]

Other abnormalities associated with poor prognosis include deletions on chromosome 17p (p53 locus).[13] Alterations of the p53 gene and p53 protein represent one of the most frequent genetic abnormalities in human cancer. Mutated p53 protein accumulates in response to cellular stress, including DNA damage and oncogene activation.[14] Wild-type p53 protein, normally present in the nucleus of the cells, has not been detected in the majority of normal cells because of its short half-life. Conversely, p53 mutant or inactivated proteins exhibit a longer half-life and they can be detected by immunologic techniques using anti-p53 monoclonal antibodies (MoAb).[15]

A simultaneous evaluation of CD38, ZAP-70 and mutated p53 protein expression allowed distinguishing the patients groups with the most favorable prognosis as well as those with the worst.[4,9,16]

So this study was conducted with the aim to estimate the clinical value of ZAP-70, CD38 and mutated p53 protein expressions as predictors of disease progression and overall survival in B-CLL patients.

II. METHODOLOGY

A descriptive analysis type of observational study was conducted on 175 patients, who were presented to Hematology Clinic, University Hospital Alexandrovskafor3 year- period, were included in the study. All patients were diagnosed according to the revised criteria of the National Cancer Institute-sponsored Working Group (NCI-WG) on Chronic Lymphocytic Leukemia (CLL) and were classified according to the Rai staging system.[17]

2.1 Definition of total tumor mass score (TTM)

TTM is the sum of: (1) the square root of the number of peripheral blood lymphocytes per ml, (2) the diameter of the largest palpable lymph node in centimetres, and (3) the enlargement of the spleen below left costal margin in centimetres.[18] The TTM>9 was related with advanced disease.

2.2 Treatment regiment

Assessments of CD38, ZAP-70 and mutated p53 protein expressions were performed in previously untreated patients with CLL. The antileukemic treatment was represented by chlorambucil+Prednisone, CVP-regimen (Cyclophosphamide+Oncovin+Prednisone), Fludarabine alone, RFC-regimen (Rituximab+Fludarabine+Cyclophosphamide) or alemtuzumab (anti CD52 monoclonal antibody).

2.3 Definition of overall survival

Overall survival (OS) was calculated from the date of initial treatment to the date of death from any cause.

2.4 Flow Cytometry

For cytoplasmic ZAP-70 staining, cells were first permeabilized by means of permeabilizing solution (Becton-Dickinson, San Jose CA). Fluorescent labeling was evaluated by flow cytometry using a FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA). The cut-off point of positivity was considered when 20% or more cells stained with the antibody for ZAP-70 expression and 30% or more positive cells for CD38 expression. The mutated p53 protein expression was estimated as the ratio between the arithmetic mean of the intensity of the fluorescence (MIF) in cell suspensions with anti-p53 MoAb and the MIF of cell suspensions labeled with the isotypic control. The samples were considered positive when the MIF ratio was greater than 1.4.

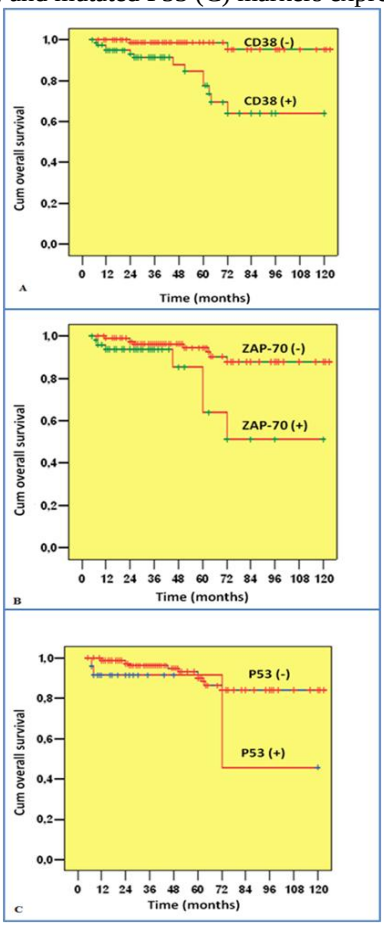
2.5 Statistical Analysis

Data analysis was carried out using trial version of Statistical Package of Social Sciences (SPSS) version 17.0.1. Qualitative data expressed in frequency and percentages was analyzed using Fisher Exact test and $\chi 2$ test. The association with overall survival was tested using the Kaplan-Meier estimator and log-rank test. A p-value < 0.05 was considered significant for all statistical calculations. A Cox proportional-hazard regression was used in multivariate analyses.

III. RESULTS

Observations were described in as follows:-

Figure 1
Kaplan–Meier curve of median overall survival time in CLL patients according to CD38 (A), ZAP-70 (B) and mutated P53 (C) markers expression



a) Biological characteristics of CLL patients: The patients enrolled in this study (105 male, 70 female) had an age range of 37-82 years (median 67 years). According to the age distribution 11% of patients were included into <50 year age group, 67% into 51-70 year age group, followed of 22% into over 70 years age group.

b) CD38, ZAP-70 and P53 expression analyses : According to the cut-off value for ZAP-70, CD38 and mutated p53 protein expression the CLL patients were divided into 2 groups: those with positive marker expression and with negative marker expression. Fifty-one patients (29%) were positive for ZAP-70, 81 patients (46%) - for CD38 and 24 patients (13.7%) were positive for mutated p53 protein expression.

c) Overall survival analyses: Kaplan–Meier curves and the Log-Rank test for overall survival rate and time to disease progression (TDP) were presented on Figure 1 and Table 1. Patients with CD38 (-) CLL cells had longer median overall survival (120 months, 95% CI 115.8–124.2), whereas CD38(+) cases had shorter OS (94.8 months, 95% CI 83.0-106.5, p<0.001) [Table 1]. Overall survival in CD38 (+) patients dropped rapidly between the 4th and 6th year from the diagnosis (Fig.1A). The similar tendency was found in ZAP-70 (+) cases. ZAP-70 (+) patients displayed a significantly shorter overall survival time (median 87.8 months vs. 114.2 months in ZAP-70 (-) patients, p<0.01), as shown in Fig. 1B. The survival function decreased gradually and slightly in the ZAP-70 (-) patients. In contrast, in ZAP-70 (+) cases survival function have been decreased dramatically, particularly between 40th and 72nd months.

Survival function showed no significantly shorter median survival time in p53 (+) patients (88.5 months vs. 111.5 months, p=0.49) compared with p53 (-) cases (Table 1 and Fig. 1C). There was registered a sharp drop in overall survival time curve in p53 (+) patients 72 months after the follow-up.

Table 1
Comparison of Characteristics of males and females

S. No.	Marker	Marker Expression	N	Median (X)	95% CL	
					Upper Limit	Lower Limit
1	CD38	Negative	94	119.98	115.80	124.16
		Positive	81	94.77	83.00	106.54
2	ZAP-70	Negative	124	114.23	108.39	120.06
		Positive	51	87.82	68.18	107.45
3	$_{P}$53	Negative	151	111.49	105.34	117.63
		Positive	24	88.46	56.43	120.49

N- Number; X- median overall survival (in months); CI – Confidential Interval

3.1 Multivariate analysis

To establish the factors for the disease progression single- and multiple binary logistic regressions were applied. We assessed the risk of disease progression rate (DPR) in CLL patients according to combined analysis of two (Table 2) and three parameters (Table 3).

Table 2
Disease progression rate (DPR) in B-CLL patients according to combined analysis of two parameters

Parameter	In combination with	DPR*	95% CI		p
			Low limit	Upper limit	
ZAP-70 (+)	CD38 (+)	23.02	6.75	78.48	<0.001
ZAP-70 (+)	TTM †>9	4.99	2.77	8.98	<0.001
ZAP-70 (+)	Age>70 years	6.51	3.09	13.69	<0.001
ZAP-70 (+)	Advanced clinical stage‡	4.73	2.62	8.53	<0.001
ZAP-70 (+)	p53 (+)	7.39	2.09	26.13	0.002
CD38 (+)	TTM>9	3.77	2.53	5.60	<0.001
CD38 (+)	Age>70 years	5.08	2.97	8.89	<0.001
CD38 (+)	Advanced clinical stage	3.82	2.52	5.81	<0.001
CD38 (+)	p53 (+)	19.61	2.54	151.45	0.004
p53 (+)	Age>70 years	3.31	1.39	7.85	0.007
p53 (+)	Advanced clinical stage	2.73	1.39	5.37	0.003

****DPR-progression rate; †TTM- total tumor mass; ‡ Advanced clinical stage- Rai III/ IV stages***

Table 3
Disease progression rate (DPR) in B-CLL patients according to combined analysis of three parameters.

Parameter	In combination with	and	DPR*	95% CI		p
				Low limit	Upper limit	
ZAP-70 (+)	CD38 (+)	TTM†	5.69	2.79	11.637	<0.001
ZAP-70 (+)	CD38 (+)	Age>70 years	8.92	3.48	22.902	<0.001
ZAP-70 (+)	CD38 (+)	Advanced clinical stage‡	5.35	2.65	10.796	<0.001
ZAP-70 (+)	CD38 (+)	p53 (+)	19.61	2.53	151.45	0.004
ZAP-70 (+)	TTM	Age>70 years	3.18	1.98	5.120	<0.001
ZAP-70 (+)	TTM	Advanced clinical stage	2.50	1.72	3.658	<0.001
ZAP-70 (+)	TTM	p53 (+)	3.65	1.60	8.332	0.002
ZAP-70 (+)	Age>70 years	Advanced clinical stage	2.99	1.86	4.823	<0.001
ZAP-70 (+)	Age>70 years	p53 (+)	4.33	1.55	12.073	0.005
ZAP-70 (+)	Advanced clinical stage	p53 (+)	3.74	1.58	8.866	0.003
CD38 (+)	TTM	Age>70 years	2.42	1.78	3.322	<0.001
CD38 (+)	TTM	Advanced clinical stage	1.99	1.60	2.487	<0.001
CD38 (+)	TTM	p53 (+)	4.67	1,58	13.860	0.005
CD38 (+)	Age>70 years	Advanced clinical stage	2.48	1.79	3.459	<0.001
CD38 (+)	Age>70 years	p53 (+)	6.64	1,64	26.841	0.008
CD38 (+)	Advanced clinical stage	p53 (+)	4.97	1.56	15.820	0.007
p53 (+)	Age>70 years	TTM	2.05	1.21	3.484	0.007
p53 (+)	Advanced clinical stage	TTM	1.74	1.19	2.556	0.004
p53 (+)	Advanced clinical stage	Age>70 years	2.02	1.18	3.469	0.010

****DPR-progression rate; †TTM- total tumor mass; ‡Advanced clinical stage- Rai III/ IV stages***

Based on analyses of DPR in CLL patients (Table 4) we were able to perform three different risk groups: high-risk group (DPR>10), median- risk group (DPR≤10) and favorable risk group (Table 4).

Table 4
Classification of CLL patients into disease progression risk groups according to CD38, ZAP-70 and mutated p53 marker expression

Risk groups	DPR*	Factors and combinations
High-risk group (DPR>10)	23.021	ZAP-70(+)/CD38 (+)
	19.606	CD38(+)/p53(+)
	19.606	CD38(+)/ZAP-70(+)/p53(+)
Median- risk group (DPR≤10)	< 10	Single ZAP-70, CD38, mutated p53 positive expression in combination with the other poor prognostic factors (age>70 years, advanced clinical stage‡, TTM†>9)
Favorable risk group	1	ZAP-70, CD38,p53 negative expression in combination with the other favorable prognostic biological factors (age<70 years, earlier stage, TTM<9).

DPR-progression rate; †TTM- total tumor mass; ‡ Advanced clinical stage- Rai III/ IV stages

IV. DISCUSSION

The objective of the present study was to assess the impact of the expression of ZAP-70, CD38 and mutated P53 protein on disease progression and overall survival in B-cell CLL.

Results of present study showed that 81 (46%) of CLL patients showed high level of CD38 expression. Similar frequencies were reported in other studies[2,4] while other reports showed low percentage of CD38 positivity.[9] The possible cause for this discrepancy may be the fact that CD38 expression can change with time and treatment. For this reason the frequency and the clinical significance of CD38 expression should be evaluated at the time of diagnosis. A comparison of median overall survival time showed significantly longer OS in CD38 (-) cases (120 months) vs. those with high level of CD38 expression (94.8 months, $p<0.001$).

As regards ZAP-70 expression, our study showed that 51 (29%) of CLL patients were positive. The frequencies of ZAP-70 expression were variable in recently published studies but in the most of them higher levels of ZAP-to were associated with advanced clinical stage and more aggressive disease.[2,4,5,9] Our data improved the negative impact of high ZAP-70 levels on overall survival and increased risk for disease progression. The median OS was significantly shorter- 87.8 months for the ZAP-70(+) cases compared with negative ones-114.2 months, $p<0.01$.

In contrast to the other techniques (PCR, Western blot or immunohisto chemistry) flow cytometry has the advantage of measuring mutated p53 protein levels in individual cells.[15,19] Cavalcanti et al.[15] demonstrated lower frequency of p53 protein expression (5.9%) in newly diagnosed CLL cases compared with advanced stage disease (88.95%). Probably, the expression of mutated p53 protein has an important role in the evolution of malignant clone in CLL. In this study 24 (13.7%) of CLL patients had mutated p53 protein expression. The presence of p53 protein expression has been associated with poor survival and failure to therapy and they were reported in patients with transformation to Richter's syndrome.[15] We reported that the presence or absence of p53 mutations showed no significant impact on OS ($p =0.49$) but p53 protein positive cases demonstrated sharp decline of survival function 72 months after the follow-up.

Data obtained in this study confirmed that CD38, ZAP-70 and mutated p53 protein as single markers have negative impact on the overall survival time. But the single expression of these markers was related with lower disease progression rate (DPR<10) and were distributed in the middle risk group.

CD38, ZAP-70 and p53 negative patients were with the lowest DPR (DPR=1) and were classified in the CLL group with good prognosis.

Morilla et al. explained that the combined analysis of ZAP-70, CD38, and IgVH mutation status showed an increased median survival in patients who were ZAP-70/CD38/IgVH mutated markers negative v/s those who were ZAP-70 positive/CD38 positive/IgVHunmutated.[20] Our survival study confirmed the negative impact of the same positive markers. CD38 (+), ZAP-70 (+) and p53 (+) patients have had 4-fold increased mortality rate then the patients with negative marker expressions and this rate was significantly higher after the 6th year of the disease beginning.

Both ZAP-70 and CD38 expression were shown to predict the clinical course of the disease and disease progression rate.[2, 3] Our study confirmed that double positive CD38/ZAP-70, CD38/P53 and ZAP-70/P53 expressions showed an increased risk of disease progression over 19-fold above the negative CLL cases ($p<0.001$). They were classified in a high risk CLL group.

Interestingly, the combination of the both CD38 and ZAP-70 markers showed the highest DPR, compared with double positive CD38/p53 or ZAP-70/p53 cases. Probably, ZAP-70 and CD38 expressions appeared to be more predictive than p53 expression and more relevant in defining the cases of B-CLL with higher disease progression rate.

At multivariable analysis, combined CD38/ZAP-70/p53 status confirmed its independent prognostic role. Triple positive cases were associated with the shortest OS and increased DPR. Thus, ZAP-70, CD38 and mutated p53 protein expression analyses allow distinguishing the patient groups with good prognosis as well as those with poor one.

V. CONCLUSION

It can be concluded from this present study that ZAP-70 and CD38 expressions appeared to be more predictive than p53 expression and more relevant in defining the cases of B-CLL with higher disease progression rate.

CONFLICT OF INTEREST

None declared till now.

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