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ORIGINAL ARTICLE

Detection of ZAP-70 in patients with chronic lymphocytic leukemia

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Summary

Purpose: *To determine the presence and prognostic significance of ZAP-70 in patients with B chronic lymphocytic leukemia (B-CLL). Expression of ZAP-70 in B-CLL is a continuum ranging from absent to high, affecting the prognostic reliability of this marker.*

Patients and methods: *32 patients with B-CLL treated from March 2006 to February 2007 were prospectively studied. Patients were stratified in two groups: those diagnosed with B-CLL within 18 months and a retrospective group diagnosed before 18 months since the beginning of this study. Patients were predominantly males (78.1%), over 60 years old (81.25%). ZAP-70 was detected on EPICX-XL flow cytometer with System II software. Directly conjugated antibodies CD19 PE, ZAP-70 FITC (clone 1E7.2) and CD2 APC were used. Leuoperm was used for permeabilization. All samples with over 20% ZAP-70/CD19 double positive B lymphocytes were considered positive.*

Results: *ZAP-70 was absent in a group of 22 patients with B-CLL diagnosed over 18 months from the beginning of this study, while 2 out of 10 (20%) patients from the group of newly diagnosed patients were ZAP-70$^+$. During follow up both ZAP-70$^+$ cases showed worsening of their clinical status resulting to death in one patient. In borderline cases with ZAP-70$^+$ ranging from 10.9-19.9% there was no correlation of the level of ZAP-70 expression with disease activity or clinical scoring systems.*

Conclusion: *Very high percentage of ZAP-70$^+$ cells is a sign of poor prognosis in B-CLL. Borderline expression of ZAP-70, although more frequent, could not be successfully assigned into a risk group.*

Key words: chronic lymphocytic leukemia, prognostic factors, ZAP-70

Introduction

ZAP-70 is a tyrosine kinase expressed and functioning in certain B lymphocytes. It is known that ZAP-70 is able to change transduction of signals from B-cell receptor in the B cells of CLL [1]. Cells in B-CLL expressing ZAP-70 have shown stronger reactivity to B-cell receptor stimulation *in vitro* compared to CLL cells without this tyrosine kinase. Therefore, presence of ZAP-70 is considered to enhance signals responsible for proliferation and to contribute to disease progression [1].

Although today there are over 40 different prognostic markers, the greatest breakthrough in the early detection of high-risk patients with B-CLL was achieved by examining somatic hypermutations of the IgVH region in B-cell receptor. Cases with hypermutated IgVH had median survival of 24 years [2], while those without hypermutation had a much poorer median survival reaching only 7-9 years [3].

Both subtypes of B-CLL (with and without IgVH hypermutation) differed from each other in the expression of several hundreds of genes in gene expression profiling analysis, but the biggest difference was in the expression of ZAP-70 gene. This tyrosine kinase was expressed in a group with worse prognosis that had no hypermutated IgVH region, while it was less expressed in a group with somatically mutated IgVH genes [4]. This was a reason to propose the ZAP-70 detection as a surrogate marker of IgVH mutational status. Knowing that the detection of IgVH is a long-lasting and labori-

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ous process compared to ZAP-70 detection by flow cytometry, use of flow cytometry for this purpose was associated with great enthusiasm in clinical practice. This was a reason to start using flow cytometry for ZAP-70 detection in our institution.

The aim of this study was to estimate the presence and prognostic significance of ZAP-70 in patients with B-CLL.

Patients and methods

This prospective study was carried out on 32 patients with B-CLL diagnosed and treated at the Clinic of Hematology and Clinical Immunology Nis, Serbia. Only cases previously diagnosed with typical B-CLL immunophenotype according to scoring criteria given by Matutes et al. were selected [5]. All patients gave written informed consent according to the Helsinki criteria. Patients were followed from 01.03.2006 until 28. 02. 2007. Males predominated (78.1%; 25 males, 7 females) as well as elderly over 60 years old (81.25%). The average age was 65.5 years (range 38-80).

Patient stratification

Patients were stratified in a group with B-CLL diagnosed within 18 months from the beginning of the study (10 patients) and in another one with disease diagnosed more than 18 months before the start of this study (22 cases; Table 1).

ZAP-70 determination

ZAP-70 was determined on EPICX-XL flow cytometer, with System II software (Beckman Coulter). The following directly conjugated monoclonal antibodies were used: CD19 PE, ZAP-70 FITC (clone 1E7.2, eBioscience, San Diego, Calif, USA) and CD2 APC (Becton Dickinson, Pharmingen, San Jose, USA). Fix and perm reagent Leuoperm (Serotec, Kidlington, UK) was used for permeabilization. T lymphocytes of the patients were used as positive control.

Flow cytometry analysis was done as previously described by Crespo et al. [6]. Briefly, in 100 µl of whole blood, 5 µl CD19 PE and 5 µl CD2 APC were added. After 10 min of incubation, a permeabilization reagent A (100 µl) (Serotec, Kindington) and 5 µl ZAP-70 FITC were added. According to the manufacturer's instructions, when the second incubation period expired (15 min), the 100 µl of reagent B (Fix and Perm) were added. The sample was washed in PBS and the remaining pellet was resuspended in 2% formalin and PBS solution. The sample was then analyzed on the flow cytometer. Gating was performed on lymphocytes. Double positive ZAP-70/CD2 T lymphocytes were also used as a positive internal control. Samples with over 20% of $ZAP\text{-}70^+/CD19^+$ cells were considered positive. In case of existence of two $ZAP\text{-}70^+/CD19^+$ populations, the population with smaller side and forward scatter was chosen in order to avoid reading signals from the artificially created doublets. At least 50 000 events were used for interpretation. For the determination of positivity the Caltag's percentage method was used according to instructions given elsewhere [7].

Statistical analysis

Statistical analysis of the data was done using the Statistica 5.0 software. x^2 test, Mann-Whitney sum range test and Spearman rank correlation test were used for analysis of non parametric data, while Student's t-test was used for parametric variables.

Results

ZAP-70 detection

ZAP-70 was detected in a very low percentage of malignant B lymphocytes in the group of patients with a diagnosis made more than 18 months since the beginning of this study.

They all had less than 10% $ZAP\text{-}70^+/CD19^+$, except 5 cases with 11.9% positive cells; none of those patients showed signs of disease progression during follow up (Table 1).

Table 1. Clinical characteristics and ZAP-70 distribution among the 2 groups of patients

Characteristic	*Group I* (n=22) n (%)*	*Group II** (n=10) n (%)*	*p-value*
Age, years (X ± SD)	65.5 ± 9.74	63.8 ± 13.6	n.s.§
Male: female ratio	1:4.5	1:2.33	n.s.†
RAI stage			
< 2	13 (59)	8 (80)	n.s.‡
3	5 (23)	1 (10)	n.s.‡
4	4 (18)	1 (10)	n.s.‡
ZAP-70 (%)			
<10	17/22 (77.27)	4/10 (40)	p=0.03†
10.9-19.9	5/22 (22.72)	4/10 (40)	
>20	0	2/10 (20)	

*diagnosis made >18 months since the beginning of this study, **diagnosis made ≤18 months since the beginning of this study, SD: standard deviation, n.s.: non significant, ‡Mann-Whitney Sum range test, †x^2 test, §Student's t-test

In the group of patients with B-CLL diagnosed within 18 months from the beginning of the study, 2 out of 10 samples (20%) had 20% ZAP-70^+/$CD19^+$ B lymphocytes (Figure 1). During follow up, both ZAP-70^+ patients showed disease progression with lethal outcome in one case. In the remaining 8 patients of this group ZAP-70^+/$CD19^+$ positive cells were less than 10% in 4 cases and in 4 cases ranged between 10.9-19.9%. Using x^2 test we observed that the expression of ZAP-70 was higher in 4 cases belonging to the group of patients diagnosed within 18 months since the beginning of the study (Table 1), while the other 4 cases had no signs of disease activity; in the latter 4 patients disease activity and clinical stage was in discordance to the percentage of ZAP-70^+/$CD19^+$ cells. In that manner for example, a case with 12.6% of ZAP-70^+/$CD19^+$ cells had Rai IV clinical stage, while a case with 15.5% ZAP-70^+/$CD19^+$ cells was in stage I with $188 \times l0^9$/L lymphocytes. A third case had 17.7% ZAP-70^+/$CD19^+$ and was Rai stage 0.

Clinical prognostic parameters

There were 6 cases in stage III and 5 in stage IV according to Rai. None of those patients had >20% ZAP-70^+ B-lymphocytes. In those cases with advanced disease stage Spearman rank test found no correlation between levels of ZAP-70 expression and stage of disease according to the Rai scoring criteria. B symptoms, worsening of performance status, progression of lymphadenopathy, newly developed organomegaly and transformation into a higher stage during prospective and retrospective follow up was found in 6 cases. Among them were 2 patients with >20% ZAP-70^+/$CD19^+$ B-lymphocytes. High level of serum LDH was registered in 10 patients with 3 cases suffering of autoimmune hemolytic anemia. The follow up period was too short for detection of lymphocytes' doubling time in all cases. Spearman rank test found no correlation between disease activity and clinical stage at the moment of analysis (p=0.07).

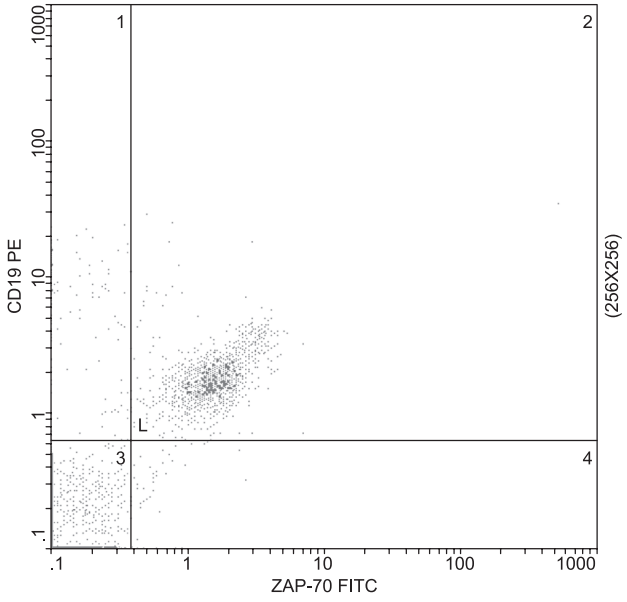

Figure 1. Double positive CD19 PE and ZAP-70 FITC cell population in a patient with aggressive form of B-CLL (shown in the L2 quadrant). There were 38% of ZAP-70^+ malignant B lymphocytes. The patient died within 16 months since the beginning of the disease. 1, 2, 3 and 4 in the angles of the rectangle represent the numbers of the quadrants divided with line marked with letter L. Values on the horizontal and vertical coordinates from -1-1000 represent the intensity of the fluorescence signal picked by the photomultiplicator of the flow cytometer.

Discussion

Flow cytometric detection of ZAP-70 could vary considerably, depending on the type of the antibodies used, method of doing the assay and the condition of the cells present in the sample. In a study by Crespo et al. [6] tests were done on frozen lymph node tissue samples. In this model, ZAP-70 expression could successfully predict the clinical disease activity in patients with Binet stage A but not in stages B and C. A study of Sheikholesami et al. [8] was done on fresh samples clearly showed that ZAP-70 is not robust but, on the contrary, a very sensitive test that requires careful interpretation. The authors found that raising the concentrations of added ZAP-70 antibody to the sample make the percentage of positivity higher, transforming negative cases into positive ones. Sheikholesami et al. also found that the level of staining and permeabilization could vary from sample to sample. The study by Best et al. clearly showed that use of heparin displayed minor variations in result interpretation in comparison to EDTA [7]. Therefore, it is still recommended not to make clinical decisions based solely on the results of detection of ZAP-70 protein [6].

One of the ways to overcome these disadvantages is to use isotype control or internal control in the form of residual T and natural killer (NK) lymphocytes that are constitutively ZAP-70^+. In the first occasion, ZAP-70^+ cases are considered all samples where the percentage of fluorescent cells is higher compared to the isotype control. In the second setting, a sample is considered positive if the percentage of positive fluorescent cells is comparable with the percentage of positive T and NK lymphocytes. The isotype control was shown to have low cut off value, based on which the expression of ZAP-70 is determined. Goolsby et al. found that T cells of patients with B-CLL are significantly different than

normal T lymphocytes [9]. Herishanu et al. also found that the level of ZAP-70 expression is significantly higher in T lymphocytes with B-CLL compared with normal controls [10]. It could be summarized that there is no method for detection of ZAP-70, which could be considered reliable for definition of negative control. This fact enabled us to make the method of ZAP-70 detection simpler, by not using an isotype control during the measurement process. Meanwhile, the method with blocking antibodies showed high specificity and accuracy concerning positive and negative cases. Although more demanding, this method has a great chance to become standard for the determination of expression of ZAP-70 in CLL [11].

Criteria for considering a B-CLL patient as ZAP - 70^+ vary, giving positive results from 10 to 20% [6, 12,13].

In our study the Caltag's percentage method was used in order to determine positivity, although lately some other methods based on staining population relations have appeared [7].

Both our positive cases had over 30% ZAP-70^+ B lymphocytes and this was also confirmed with their unfavorable clinical course. The remaining cases were not homogeneous in respect to their clinical course as well as in respect to the recognized clinical scoring systems. Owing to these discrepancies some authors suggest changing the cut off value to define high-risk patients from 20 to 26% ZAP-70^+ cells [14].

On the other hand, numerous cases of CLL do not have so high percentage of ZAP-70^+ cells. In fact, the study of Rassenti et al. has shown that the expression of ZAP-70 is a continuum, ranging from 0 to very high percentages [13]. Most of the technical mistakes appear in cases where the percentage of expression is close to borderline. Several borderline cases were registered in our study. They still represent a grey zone and many authors are aware of the numerous and still unsolved pitfalls of ZAP-70 detection. This is the main reason for not classifying borderline cases into a negative group. Their correct place would be found as soon as all technical difficulties are overcome.

The fact that ZAP-70 was higher in the group of patients' diagnosed $\leq$ 18 months since the beginning of the study compared to the other group, could be also a result of a possible selection bias. In fact ZAP-70^+ patients have inferior survival, and therefore they might already have die, leaving only cases with low expression of ZAP-70 available for inclusion onto study, in the group where diagnosis had been made >18 months since the beginning of this study.

We conclude that ZAP-70^+ exceeding 20% could be considered as an adverse prognostic factor. Expression of ZAP-70 in CLL malignant lymphocytes could be continuous, ranging from minimal to very high, which makes its prognostic significance less accurate, especially in borderline cases. More reliable conclusions could be drawn only in studies with higher number of patients and longer follow up.

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