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Clinical Science

The Diagnostic Value of Flow Cytometry Imunophenotyping in an Albanian Patient Population with a Preliminary Clinical Diagnosis of Chronic Lymphocytic Leukemia

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Abstract

Objective: Based on the flow cytometry multiparametric immunophenotyping methodology we studied some useful cell marker criteria needed for the practical differentiation of the chronic lymphocytic leukemia from other chronic limphoproliferative diseases with a leukemic component.

Materials and Methods: The applied methodology is a four color flow cytometry multiparametric immunophenotyping technique using EDTA blood samples taken from 84 consecutive patients diagnosed with CLL through a preliminary clinical and white blood cell examination. The following fluorescent stained monoclonal antibodies were used: CD3, CD4, CD5, CD8, CD11c, CD19, CD20, CD23, CD25, FMC7 and kappa/lambda light chains.

Results: From the 84 individuals tested, 2 out of them (2.4%) resulted with a abnormal T-cell population while 82 (97.6%) showed a pathological B cell line. 58 (69.1%) patients resulted with typical CLL markers (CD19+CD5+CD23+) while 5 (5.9%) of them presented a non typical chronic lymphocytic leukemia profile (CD19+CD5+CD23-). 19 (22.6%) out of patients displayed an abnormal CD19+CD5- B cell population. A statistically significant correlation was found between the clinical stage of CLL and the positivity for the CD38 marker (p=0.04).

Conclusion: Flow cytometry immunophenotyping is a fundamental examination for the final diagnosis of chronic lymphocytic leukemia. The expression of CD38+ in CLL patients stands for a more advanced clinical stage.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in adults [1]. It is generally accepted that any lymphocytosis superior to 5000/mm^3 in an adult person, and without any other evident cause, must raise the suspicion of a CLL diagnosis [2]. The leukemia cells found in the CLL blood smear are characteristically small, mature lymphocytes with a narrow border of cytoplasm and a dense nucleus lacking discernible nucleoli and having partially aggregated chromatin [3]. The patients presented with clinical and laboratory signs evoking CLL must be thoroughly checked if they have or not some other chronic lymphoproliferative disease (CLPD) that can masquerade as CLL, such as hairy cell leukemia, leukemic manifestations of mantle cell lymphoma, marginal zone lymphoma, splenic marginal zone lymphoma with circulating villous lymphocytes, or follicular lymphoma [4, 5]. In order to achieve this, it is essential to evaluate the blood count, the blood smear, the detailed immunophenotype of the concerned lymphoid cells and often additional immunohistological or cytogenetic studies are also needed [6, 7]. The purpose of this study was to evaluate the impact of the application of the multiparametric flow cytometry immunophenotyping (FCI) as a standard testing methodology for the confirmation or exlusion of CLL diagnosis in a population of 84 consecutive Albanian patients in whom the CLL diagnosis has been set in advance on the basis of clinical, blood cell counting and peripheral blood smear cytology data. We also aimed to determine the contribution of FCI for the evaluation of

the staging and disease progression among the CLL patients.

Materials and Methods

Patients

This study was carried out at the Laboratory of Immunology and Histocompatibility of the University Hospital Center "Mother Teresa" of Tirana, Albania during the time interval January 2011 to december 2012.

It included 84 consecutive patients previously diagnosed with CLL through a preliminary clinical evaluation and a standard blood cell examination. The patient blood samples were sent to our laboratory from the Department of Hematology of the "Mother Teresa" University Hospital Center of Tirana in order to confirm the CLL diagnosis.

The age of the patients ranged from 40 to 85 years (mean: 61.2 years). 55 (65.5%) of them were males and 29 (34.5%) females. The blood samples were examined at our laboratory immediately after their arrival and the immunophenotypic diagnosis was established without any previous knowledge about the clinical or hematological data. The patient files were studied retrospectively in order to compare the flow cytometry results with the clinical data. The clinical data were used to classify the patients into different clinical CLL stages as following the Rai classification [8]. The patients who presented only a blood lymphocytosis superior to 5000/mm^3 and/or a bone marrow lymphocytosis (lymphoid cells >30%) were defined as having low-risk disease or Rai stage 0. Patients with lymphocytosis, enlarged nodes in any site, splenomegaly and/or hepatomegaly were defined as having intermediate-risk disease (Rai stage I or stage II). High-risk disease included Rai stage III and IV patients with disease-related anemia (as defined by a hemoglobin level <11 g/dL) or thrombocytopenia (defined by a platelet count < 100.000 mm^3).

Methods

The applied methodology was a four color FCI technique using EDTA blood samples that were examined in an EPICS-XL Beckman-Coulter flow cytometer. The samples were processed through a "no wash, staining and lysis" procedure using the Optilyse reagent (Beckman-Coulter, California, USA) as recommended from the manufacturer. A "lysing, wash and staining" procedure was applied only for kappa and lambda light chain markers. The FCI was carried out through a gating strategy based on the side scatter (SS) versus CD45 white cell scattering (Fig. 1). The following stained monoclonal antibodies directed to the membrane cell marker antigens were used for the white blood cell staining with this method: CD3-PC5, CD4-PE, CD5-PE, CD8-ECD, CD11c-PE, CD19-PC5, CD20-PE, CD23-ECD, CD25-ECD, CD38-PC5. The membrane cell marker FMC7 and kappa/lambda immunoglobulin light chains were examined through a two color cell staining and by gating on the lymphocyte population as following a SS versus forward scatter (FS) cell distribution. If more than 20% of the gated cell population resulted positive for a given stained monoclonal antibody it was considered as positive for the respective cell marker. The Fischer exact test was used to evaluate the differences between the categorical variables.

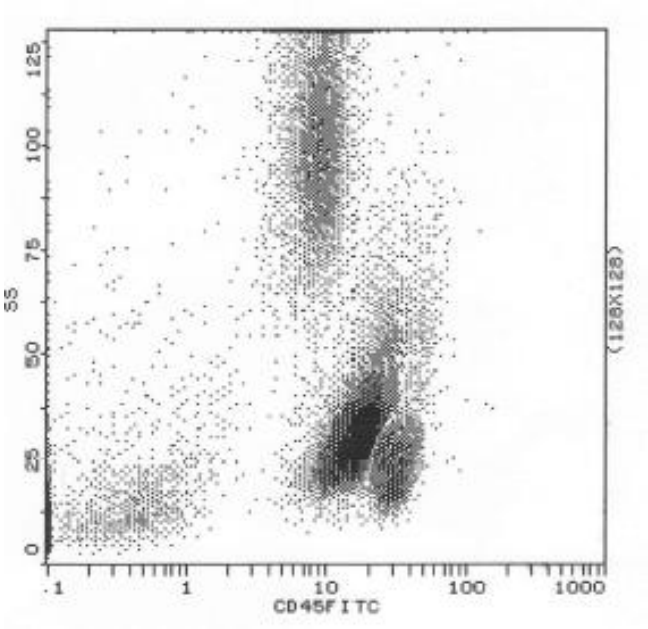

Figure 1: Flow cytometry CD45 versus side scatter (SS) in CLL cell differentiation.

Results

Diagnostic differentiation of the abnormal lymphoid cell population through FCI

The cell marker positivity results observed on the abnormal cell populations of all the patients studied is presented in Table 1.

Table 1: The cell markers studied and their positivity rate on the leukemic cells of all the patients examined.

Leukemic cell markers studied (No.of patients tested)	Marker positivity No.of positive patients (%)
CD3 (84)	2 (2.4%)
CD4 (84)	2 (2.4%)
CD5 (84)	65 (77.3%)
CD8 (84)	1 (1.2%)
CD11c (20)	3 (15.0%)
CD19 (84)	82 (97.6%)
CD20 (84)	45 (53.6%)
CD23 (84)	63 (75.0%)
CD25 (22)	3 (13.6%)
CD38 (35)	18 (51.4 %)
kappa/lambda light chains (23)	16 (69.6%)
FMC-7 (18)	3 (16.6%)

From all the 84 individuals tested, 2 of them (2.4%) resulted with an abnormal T cell population (CD3+, CD4+ and/or CD8+) while the resting 82 (97.6%) of patients showed a pathological B cell line. From these, 58 (69.0%) out of all patients resulted

with a typical CLL cell marker profile (CD19+CD5+CD23+) whilst 5 (6 %) of them showed a non typical (CD19+CD5+CD23-) CLL profile. An abnormal CD19+CD5- B cell population has been observed in 19 (22.6%) out of patients. Among these, 3 (3.6%) resulted with hairy cell leukemia (HCL) markers (CD19+CD5-CD23-CD11c+FMC-7+). In Table 2 are shown the results of the CD20 marker positivity among the B cell CLPD diagnosed according to their classification after the FCI examination. The positivity rate of this cell marker has been found higher in both non typical CLL and CD19+CD5- patient subgroups in contrast to the typical CLL patients but a statistically significant difference was found only between the typical CLL and the CD19+CD5- group of patients (P < 0.001).

Table 2: CD20 marker positivity among the B cell CLPD according to their classification after the FCI examination.

	Typical CLL	Atypical CLL	B-CLPD (CD5-)
CD cell marker positivity	CD19+CD5+CD23+ (n = 58)	CD19+CD5+CD23- (n = 5)	CD19+CD5-CD23- (n=19)
CD20 +	44,8%	20 %	93,7%

CLL = Chronic lymphocytic leukemia; B-CLPD = B-cell chronic lymphoproliferative diseases.

Correlation studies of the immunophenotypic markers with the disease progression

The classification according to RAI stratification of the typical CLL patients of our series is shown in Table 3.

Table 3: Classification of typical CLL patients according to the clinical stages by RAI.

CLL Stage	Number of patients (%)
0	0 (0 %)
I	6 (10.3%)
II	18 (31.03 %)
III	20 (34.4 %)
IV	14 (24.13 %)
Total	58 (100 %)

We investigated also for the presence of any association between the CD38, CD20 and immunoglobulin light chain membrane cell markers and the disease progression (Table 4). A statistically significant association was found only between the CLL clinical stages and the positivity for the CD38 marker (P = 0.04).

Table 4: Correlation between the positivity of the cell markers CD38, CD20, kappa/lambda light chain and the disease stages among the patients with typical CLL immunophenotypic profile.

Marker positivity	Stage 0, I, II	Stage III - IV	P – Value
CD38+	2 (25%)	14 (70%)	P = 0.04
CD20+	12 (50%)	14 (41.1%)	P = 0.60
light chain+	3 (75%)	8 (72.7%)	P = 1.00

Discussion

Although it is well known that CLL is the more common cause of leukemia among adults older than 50 years, many other malignant CLPD can mimick CLL when they are accompanied by a leukemic cell component (9). In addition to the classical cytology, several diagnostic tools such as FCI, immunohistochemistry or cytogenetic and molecular biology methods are needed in order to establish a precise CLPD diagnosis [10, 11]. However, in health care settings with limited financial and technological resources, it is often difficult to implement all these methods concomitantly, due to their high cost and also the need for well trained and skilled personnel Taking into account the fact that multiparametric FCI has been shown as a practical, rapid and powerful tool for the diagnosis of malignant hemopathies, we studied the impact of the implementation of this methodology into the more precise diagnosis of 84 consecutive patients sent at our laboratory with a preliminary CLL diagnosis established on the basis of their clinical and cytological data.

From the 84 consecutive patients with a preliminary CLL diagnosis, 2 of them (2.4%) resulted with a blood T-cell leukemic component. Some authors do not accept the diagnosis of T-cell CLL and according to them the most likely diagnosis in this situation is the prolymphocytic T-cell leukemia or the Sezary síndrome with a leukemic component [12-14]. The presence of the marker CD5 is a prerequisite condition for the diagnosis of CLL [15, 16]. For this reason we divided the patients presenting an abnormal B cell population into the CD5+ and CD5- subgroups. Into the CD5+ subgroup were included 63 individuals or 76.8% of all patients initially diagnosed as CLL.

According to the literature reports the leukemic CLL B cells typically express the three characteristic markers CD19+, CD5+ and CD23+ [17, 18]. In our study they accounted for 92.0% of CD19+ CD5+ patients or 69.1% of all the patients studied. So, the typical CLL in our study constitutes no more than 70% of all our patients presenting a CLPD associated with a leukemic blood cell population. The patients with an abnormal CD5+ CD19+ CD23- cell population constituted 5.9% of all the individuals studied or 8% of CD19+ CD5+ patients. Like other authors we named this group as atypical CLL [19] in contrast with the typical CLL patients who show the CD5+ CD19+ CD23+ immunophenotype.

The CD5+CD19+CD23- immunophenotypic profile is evocative also for the mantle cell lymphoma (MCL), the chronic prolymphocytic leukemia or eventually another B –cell CLPD [20, 21]. The accurate MCL diagnosis requires a further and more detailed imunohistochemistry and cytogenetic study including other markers such as Cyclin D1 or 11, 14 chromosome translocations [22, 23]. MCL abnormal cells often express also the FMC7 marker (24). In our series the FMC7 marker resulted positive in one CD5 + CD19 + CD23- patient who was later confirmed with the MCL diagnosis.

A CD19+CD5- immunophenotyping profile

was found in 22.6% of our patient group. This profile suggests the presence of a non-CLL B-cell CLPD such as hairy cell leukemia (found in 3.6% of our patients), or any other non-Hodgkin B-cell lymphoma type with a leukemic component (follicular lymphoma, marginal zone lymphomas or other large cell lymphomas) that accounted for 19% of our patient population.

The CD20 marker is weakly expressed or even absent in typical CLL cells [25]. In our study the CD20 positivity rate was 20% at the typical CLL cells and 46.6% at the atypical CLL cells. We also observed a highly significant CD20 positivity at the CD5+CD19- patient subgroup compared with the CD19+ CD5+ patients. In this regard, the CD20 can serve as an immunophenotipic marker for the differentiation of typical from atypical CLL, and also from any other type of non-Hodgkin's lymphoma.

Another interesting marker for the study of CLL is CD38 that has been associated with a more advanced disease progression and a rather poor prognosis [27-29]. We found that 70% of CD5+CD19+CD23+ positive patients expressing the CD38+ marker belonged to the disease stages III-IV, while 75% of them who did not express this marker belonged to the stages 0-I-II (P = 0.04). According to the reports from the United States of America and European countries most of the patients with an initial CLL diagnosis are currently classified at the initial 0, I and II disease stages [30]. In this regard it is worth mentioning that we have encountered in our patients a more frequent presence of disease stages III and IV (69.0%) compared to the stages 0, I and II (31.0%). This seems to be related to a rather advanced stage of the disease at the time of diagnosis.

We can conclude that FCI is a fundamental laboratory examination for the final CLL diagnosis and this diagnosis can not be established without a detailed flow cytometry immunophenotyping profile of the leukemic cells. Additional methods such as immunochemistry or cytogenetic studies must be applied when a conclusive diagnosis can not be achieved through FCI.

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