

Original Article

MDR-1 expression and soluble HLA-G levels in B-chronic lymphocytic leukemia: an impact on prognosis

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Abstract: Background: Negative expression of some poor prognostic markers like ZAP-70 and CD38 in CLL patients failed to show good outcome of the disease which lead to investigate other factors which would affect the disease behavior. Aim of the work: To evaluate prognostic impact of MDR-1 expression and soluble HLA-G level in B-CLL in Zap70 and CD30 negative patients. Methods: MDR-1 expression was measured by flow cytometry and soluble HLA-G level by ELISA in 45 B-CLL patients with ZAP-70 and CD38 are less than 20% and 30% at baseline respectively then we assessed the correlation their expression with the response to treatment. Results: Our results showed despite there was a higher level in the mean percentage of MDR-1 in the CD5⁺ CD19⁺ cells in patients who achieved partial remission (PR) compared to patients in complete remission (CR) but it was not statistically significance. On the other hand, the Mean Fluorescence Intensity (MFI) of the expression of MDR-1 was significantly higher in patients with PR compared to patients with CR (P=0.04). Regarding the soluble HLA-G there was no significant difference in its baseline expression between patients who achieved either PR or CR. Conclusion: Our findings support the possibility of considering MDR-1 using its MFI as a prognostic marker. However, the level of soluble HLA-G brings no additional prognostic value.

Keywords: MDR1, HLA-G, CLL, Pgp, flow cytometry

Introduction

Chronic lymphocytic leukemia (CLL) is heterogeneous disease. The currently available clinical staging system for CLL is simple and inexpensive but lack accuracy to predict disease progression and survival on an individual basis [1].

Novel biological markers such as ZAP-70 and CD38 expression have shown to offer important prognostic information. Both CD38 and ZAP70 expressions are traditionally evaluated by flow cytometry. After the landmark paper by Damle et al. [2], determination of CD38 expression and IgVH mutation status moved into the forefront as laboratory tests that can provide valuable data for CLL patients and some studies showed its increased expression may herald progression of disease [3]. The question of multidrug resistance (MDR) in B-CLL has attracted considerable attention in some studies

[4]. One of the best known MDR-mechanisms is linked to the over expression of membrane Pglycoprotein (Pgp), which plays a physiologic role as a pump catalyzing the rapid efflux of cytotoxic drugs from the cell and may act as an antiapoptotic molecule by reducing chemotherapy induced apoptosis [5]. As the normal cell counterpart of the B-CLL lymphocyte also expresses Pgp molecules at a low level [6], it has become evident that when the cell Pgp phenotype is studied, not only the percentage of the positive cell population but also the level of intensity of its expression should be taken into account [7].

HLA-G is a nonconventional HLA class I molecule expressed on trophoblast cells and thus protecting fetus from immunorecognition during pregnancy [8]. HLA-G exerts multiple immunoregulatory functions; HLA-G expression in tumor cells may favor their escape from antitumor immune responses thus allowing tumor

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progression [9]. The expression of HLA-G was reported in CLL, its use as prognostic factor is controversial however some reseaches suggested it could be used as novel prognostic factor [10].

As altered expression of the MDR-1 which lead to resistance to treatment also the expression of soluble HLA-G level lead to dis-regulation of important immunomodulatory role in tumorcells. So our aim was to investigate these markers in a group of patient who had negative expression of bad prognostic factors namely Zap70 and CD38 and to correlate their expression with the response to the chemotherapy.

Materials and methods

Patients

A prospective study included 45 patients B-CLL patients who treated in South Egypt Cancer Institute (SECI), Assiut University and Health Insurance Hospital, Assiut, Egypt from January 2008 to January 2013, All patients received Fludarabine, Cyclophosphamide regimens (FC) with fludarabine 25 mg/m² intravenously daily for 3 days and cyclophosphamide 250 mg/m² daily for 3 days cycle repeated every 28 days. The Patients who achieved complete response after 4 cycles were given 2 additional cycle of chemotherapy, otherwise a maximum of 8 courses were given [11].

Age, gender, Rai stage, white blood cell count, hemoglobin concentration, platelet count, serum activities of Lactate Dehydrogenas (LDH) were assessed in all patients for accurate staging. Response criteria were assessed by the NCIWG [12]. Ethical approval for this study was granted by the Institutional Review Board of SECI. All patients signed informed consent according to institutional guidelines.

In each patient morphologic diagnosis of B-CLL was confirmed by flow cytometry revealing a typical CD19⁺ CD5⁺ CD23⁺ CD10^{-ve} FMC7^{-ve} and dim Ig.

Only patients with negative ZAP-70 and CD38 and with no cytogenetic abnormalities (17 p, 11q del) were enrolled into this study.

Monoclonal antibodies: For immunostaining and analysis by fluorescence-activated cell sorting (FACS), we used allophycocyanin (APC),

peridinin chlorophyll protein (PerCP), and fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibodies (mAb) against human CD19, CD38 and CD5 respectively and the corresponding mouse isotype controls (all from BD PharMingen, SanDiego, CA). Cells were stained intra-cellularly with phycoerythrin (PE)-conjugated MDR-1 (Pgp) (Becton Dickinson, San Jose, CA) in one tube and stained surfacely with ZAP-70-PE in another one, and the corresponding IgGs were used as isotype controls. All antibodies were used at concentrations titrated for optimal staining.

Four colors staining of monoclonal antibodies were carried out using a standard protocol.

Flow cytometry analysis

Flow cytometry was performed with the FACS Calibur system (BD, San Jose, CA). All fluorocytometric data were subsequently analyzed and displayed with CELL QUEST software (BD, San Jose, CA). Each analysis included measurements from a minimum of 20,000 cells.

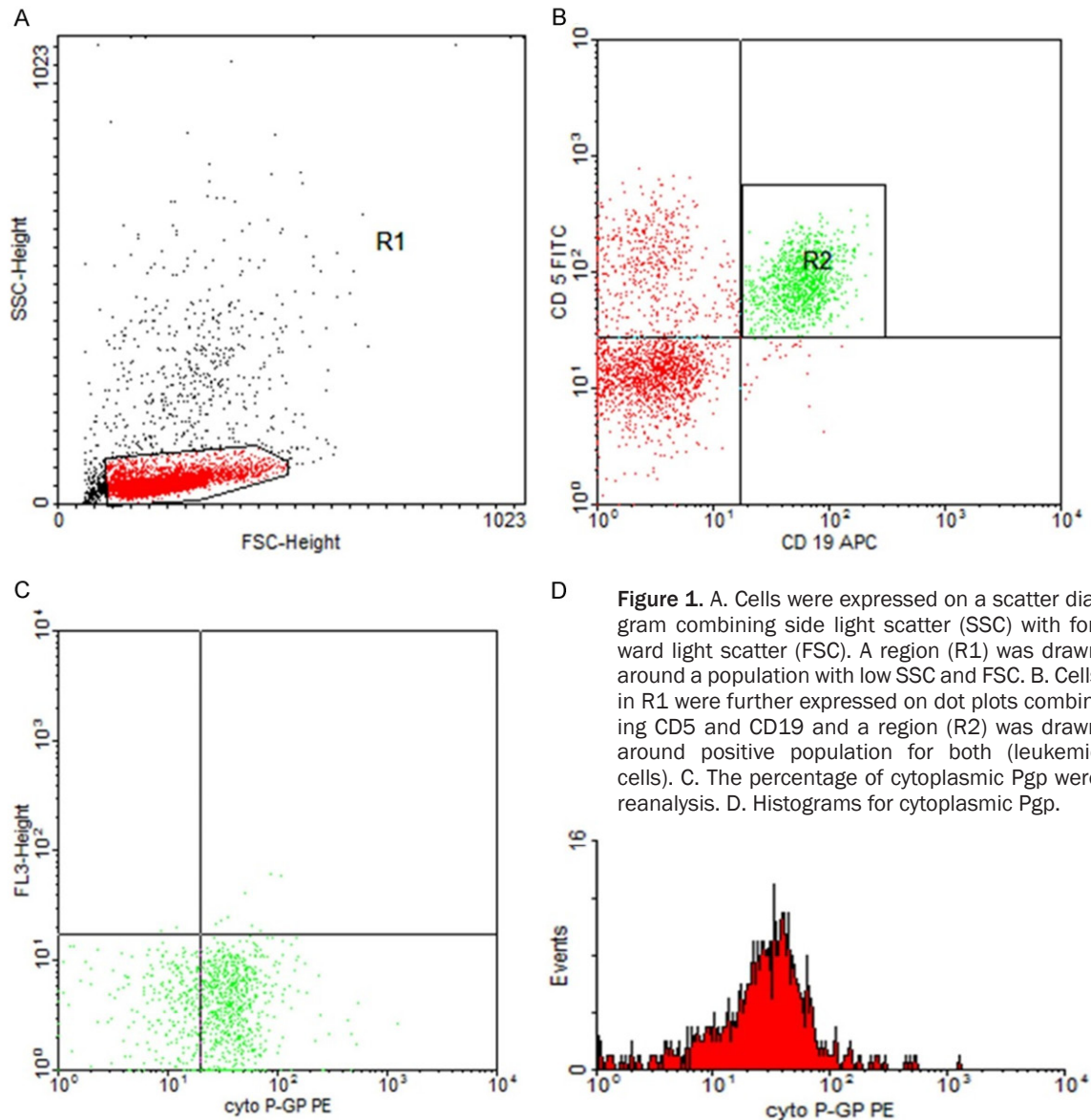
Cells were expressed on a scatter diagram combining side light scatter (SSC) with forward light scatter (FSC). A region (R1) was drawn around a population with low SSC and FSC. Cells in R1 were further expressed on dot plots combining CD5 and CD19 and a region (R2) were drawn around positive population for both (leukemic cells). The percentage of positive cells for CD38, cytoplasmic Pgp and ZAP-70 were recorded. Histograms for cytoplasmic Pgp were analyzed compared with their isotypic controls. Cell populations were interpreted as immunoreactive for a given antibody only when they showed unequivocal separation from the negative controls (**Figure 1**). The sample was considered "positive for ZAP-70 expression" based on published literature if the expression more than 20% [13].

We considered CD38 expression in greater than 30% leukemic cells as positive [2].

Soluble HLA-G (sHLA-G) levels

Levels of sHLA-G molecules (G1 and G5 isoforms) in plasma samples were determined by ELISA by using a double monoclonal sandwich enzyme immunoassay kit according to the manufacturer's instructions (Exbio).

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Statistical analysis

The data were collected, categorized and processed by using Statistical Package for Social Sciences (SPSS), version 17 software packages. The quantitative variables were expressed as mean \pm standard deviation (SD) and comparison was done using students *t*-test. *P*-value levels of <0.05 was considered statistically significant. Correlations between quantitative variables were done using Pearson correlation and multiple regression analysis by stepwise method.

Results

Forty five B cell patients were included in the study from January 2008 to January 2013, 25

patients (55.5%) were males and 20 patients (44.4%) were females. Their age range [42-65 years] mean \pm SD age 52 ± 9.5 years, the disease characteristics and laboratory data of all patients were illustrated in (Tables 1, 2).

The proportion of peripheral blood CLL cells (CD19⁺ CD5⁺) before treatment varied from 13.3% to 97.6% (55.5 ± 25.9). MDR-1 (level and intensity) and sHLA was studied in all patients before treatment.

After median 6 cycles of chemotherapy 24 (53%) patients achieved CR and 16 (35.5%) patients achieved PR, 3 (6.6%) patients had stable disease and 2 (4.4%) patients had progression. Due to small number of patients who

Table 1. Clinical data of the patients

Variables	No of patients (%)
Sex	
Male	25 (55.5)
Female	20 (44.4)
B symptoms	25 (55.5)
Rai stage	
0	0
I	14 (31)
II	15 (33)
III	11 (24)
IV	5 (11)
Remission status	
CR	24 (53)
PR	16 (36)
SD	3 (7)
PD	2 (4)

CLL: Chronic lymphocytic leukemia, CR: Complete remission, PR: Partial Remission, SD: Stable Disease, PD: Progression of the disease.

achieved stable and/or progression disease, the *P* value was done between patients who achieved CR and PR only.

We found the mean level of MDR 1 at baseline in patients achieved CR was (12.7±16.5) which was lower than patients achieved PR (23.4±22.0); SD (24.1±22.0) and PD (24.9±23). But it did not show significant difference between CR and PR (*P*<0.09). On the hand, we found Mean Fluorescence Intensity (MFI) of the expression of MDR-1 was (66.9±91.1) in CR patients, it was also lower than patients who achieved PR (75.3±58.5) SD (76.3±60.1) and PD (77.9±60.9) but we found here statistical significant difference between CR and PR (*P*<0.04) (**Table 3**).

Regarding the significant of sHLA-G, its level at baseline was little lower in CR patients (68.4±39.5) than PR (88.6±60.2) SD (90.1±61.9) and PD (92±67.5) but didn't reach the statistical significant (*P*=0.54) when compared between CR or PR (**Table 3**).

Concerning the correlation between their level and other disease parameter, MFI of MDR-1 showed moderate correlation with LDH level (*r*=0.45; *P*<0.1). While, sHLA-G had positive correlation with percentage of leukemic cells (*r*=0.59; *P*<0.05), WBCs count (*r*=0.5; *P*<0.01) and clinical stage (*r*=0.628; *P*<0.02).

Discussion

A quick review of the literature identified over 35 different prognostic markers. Some of these are classified as prognostic "traditional", whereas others are classified as "novel". The traditional prognostic factors tend to be those obtained from routine history, physical examination, and lab work. The novel prognostic factors tend to assess molecular aspects of the CLL cell themselves [14].

Biological markers such as CD38 and ZAP-70 expression have shown to offer important prognostic information. However, predicting subsets of patients who will response to a given therapy should be useful to avoid unnecessary toxicity and, theoretically at least, avoid the emergence of clones resistant to treatment [15-18]. So we tried to investigate impact of expression of MDR-1 and sHLA-G on the response of the disease to chemotherapy. We found expression of MDR-1 in Zap70⁺ and CD38⁺ patients, which is matched with the results of Guillaume N et al. [19], which established that ZAP-70/CD38 expression is not associated with a MDR1 to B-CLL cells, and that the poor responsiveness to chemotherapy of ZAP/CD38 CLL is not connected with an MDR1 mechanism.

The percentage of MDR-1 level in CLL cells was higher in patients who achieved PR compared to patients achieved CR. This finding seems to be correlated with previous studies on MDR-1 expression in CLL [20-22]. The MDR-1 polymorphism was previously described to be involved in immunological mechanism that may modify the survival [23]. Although it didn't show statistically significant that probably due to the small numbers of patients, However we found significant differences in mean fluorescent intensity of MDR-1 between patients who achieved PR than those with CR, but nobody has investigated the relation between the MFI of MDR-1 and other factors. So we recommend another large study to confirm this observation.

Regarding sHLA-G, we aimed to evaluate the sHLA-G in CLL patients to determine its prognostic value, as previous work by Nuckel et al. [24] who found superior prognostic value of HLA-G when compared with other prognostic markers in CLL. And there was growing evidence that soluble HLA molecules including

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Table 2. Patients laboratory data

Variable	Min	Max	Mean	SD
WBCs ($\times 10^9/L$)	6.90	325	103.0	88.4
Hb (g/dL)	5.60	13	10.0	1.9
Platelets ($\times 10^9/L$)	64	276	145.7	54.8
Lymphocytes in Pb ($\times 10^9/L$)	54.8	300	96.4	85.2
Lymphocytes in BM (%)	40	94	82.9	14.7
LDH	229	1051	625.1	270.4
Bone marrow cellularity (%)	42	95	79.5	11.4

CLL: Chronic lymphocytic leukemia; Min: Minimum; Max: Maximum; SD: Standard deviation; WBCs: White blood cells; Hb: Hemoglobin; Pb: Peripheral blood; BM: Bone marrow; LDH: Lactate dehydrogenase.

Table 3. Expression of Pgp in CD19⁺/CD5⁺ cells and sHLA-G

Variable	Clinical outcome	Mean \pm SD	P value*
Pgp (%)	CR	12.7 \pm 16.5	0.09
	PR	23.4 \pm 22.0	
Pgp (MFI)	CR	66.9 \pm 91.1	0.04
	PR	75.3 \pm 58.5	
sHLA-G	CR	68.4 \pm 39.5	0.54
	PR	88.6 \pm 60.2	

*Independent t test. CLL: Chronic lymphocytic leukemia; Pgp: P-glycoprotein; MFI: Mean fluorescence intensity; sHLA-G: Soluble HLA-G; CR: Complete remission; PR: Partial remission.

HLA-G might suppress immune system by forcing a pool of activated CD8⁺ T lymphocytes to undergo apoptosis [25, 26].

However our results couldn't find any significant association between base line sHLA-G plasma levels and the outcome of patients, which was equivalent with the results of Giannopoulos et al. [27] who concluded that expression of HLA-G in B-CLL on messenger level as well as its soluble form in plasma brings no additional prognostic value for B-CLL patients.

On the other hand, we found correlation with leukemic cells, WBCs count and clinical stage. This could be due to accumulate of WBCs in peripheral blood release HLA-G in soluble form and thus sustain high level of sHLA-G.

Conclusion

In summary, our results support the possibility of considering MDR-1 with using its MFI as a prognostic marker. However, the level of soluble HLA-G brings no additional prognostic value

for BCLL patients in negative ZAP-70 and CD38, and large study is warranted to support these finding.

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Disclosure of conflict of interest

None.

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