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Prognostic Value of Soluble HLA-G and CD38 Expression in Patients with Chronic Lymphocytic Leukemia in Egypt

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ABSTRACT

Background: Chronic lymphatic leukemia (CLL) is the most common hematological malignancy in adults presenting with varied clinical courses. There is an increased demand for establishing known prognostic factors for stratifying CLL patients.

Aim of the work: To investigate the expression of soluble form of human leucocyte antigen-G (HLA-G) & CD 38 in CLL and to correlate our findings with a variety of clinical and laboratory variables.

Patients and methods: The study included thirty newly diagnosed CLL patients. They were 18 males and 12 females with age ranged from 49–65 (a mean of 57.5 years old). Diagnosis of CLL was confirmed by flow cytometric immunophenotyping using standard lymphoma panel. CD38 expression was determined by flow cytometry. S. HLA-G was measured by ELISA method.

Results: Positive CD38 expression was significantly associated with lower Hb and platelets ($p = 0.008$ and 0.025 respectively) also with higher Beta2 microglobulin ($p = 0.012$). Soluble HLA G was positively correlated with the platelets count($r = 0.400$, $p = 0.02$). Higher level of soluble form HLA-G was significantly more frequent in patients of 60 years or older ($p = 0.001$). The cumulative overall survival was 83.3%. Positive CD 38 expression was associated with significantly worse survival ($p < 0.005$). HB count < 10 gm/dl was associated with significantly worse survival ($p < 0.001$).

Conclusion: CD38 expressions are considered powerful prognostic markers in predicting overall survival for Egyptian CLL patients and they should be assessed to decide the patient's therapy and to determine disease prognosis. Expression of soluble form of HLA-G in CLL patients by ELISA get no extra prognostic importance.

Keywords: CLL, HLA-G, CD38, ELISA.

INTRODUCTION

CLL is the most public form of leukemia in the world [(1)]. In Egypt, CLL was the most common subtype of leukemias and it was reported that over 80% of lymphoid leukemias are CLL [(2)].

CLL is a varied disease. The currently used clinical staging system for CLL is simple but does not predict disease advance and overall survival on an individual base. The well-recognized Rai and Binet staging systems and the well-known other prognostic markers would be unsuccessful to find B-CLL patients with disease progression who will advantage from early initiation of therapy and are the target for novel therapy [(3)]. Therefore an increased request for finding known prognostic factors is of great significance. HLA-G is a HLA class I molecule expressed on trophoblast cells and thus guarding fetus from immunorecognition during pregnancy [(4)]. HLA-G exerts multiple immunoregulatory functions. HLA-G expression on tumor cells may service their escape from antitumor immune responses, hence allowing tumor progression [(5)].

The expression of HLA-G was described in CLL, its use as prognostic factor is debatable however some scientists suggested it could be used as a unique predictive factor, CD38 is a diphosphate-ribose hydrolase that acts as a simple ecto-enzyme with adenosine diphosphate-ribosyl cyclase and cyclic adenosine [(6)]. The raised CD38 expression is associated with lower clinical outcomes of CLL [(7)].

Many laboratories now investigate CD38 expression as part of their expectable diagnostic flow cytometric analysis of CLL patients. However, the proven use of CD38 data has been concerned by an observation that major suspicions remain in the works concerning its prognostic value [(4, 5, 6)]. Some studies have stated that CD38 expression changed over time or that only moderately high levels of CD38 positivity were prognostic [(4, 5)], but another studies have shown that CD38 expression was steady over time and that the existence of even a lesser proportion of CD38-positive cells was accompanying considerably inferior clinical results.

Aim of the work: To investigate the expression of soluble form of HLA-G & CD 38 in Egyptian CLL patients and to correlate our findings with a variety of clinical and laboratory variables.

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PATIENTS AND METHODS

1- Thirty newly diagnosed B-CLL patients, 18 males and 12 females with mean age of 58.8 ± 12.1 years, were collected from the Hematology Unit of, Medical Oncology Department, National Cancer Institute, Cairo in the period from December 2017 to July 2019. Ten healthy individuals were used as control.

2- Patients were joined in prospective study and analyzed for biologic and clinical characteristics, including age, sex, presence of lymphadenopaty, organomegaly, rai stage, white blood cell count , lymphocyte count, hemoglobin concentration, platelet count and serum activities of lactate dehydrogenase, as well as serum concentrations of β_2-microglobulin, and bone marrow morphological examination, particularly lymphocyte counts.

3-Samples were taken from patients at diagnosis in addition to the 10 healthy control.

4-Our patients were categorized according to modified Rai staging system Rai *et al.* [8] into 2 subgroups:

- Low risk (stage 1 and 2): included (19) patients (63.3%).
- High risk (stage 4) included (11) patients (36.7%).

All patients were receiving the standard treatment combination of fludarabine, cyclophosphamide, and rituximab (FCR), and followed up during progress of chemothapy for 18 months for calculation of overall survival.

5-In each patient, morphological diagnosis of B-CLL was established by flow cytometry (Beckman-Coulter, Navios), with CLL-typical CD5, CD19, CD23, CD38, CD79b, FMC7 immunophenotypes, surface Ig as well as κ and λ light chain restriction. Labelling was done with fluorescein isothiocyanat (FITC) or phycoerythrin (PE).

CLL was distinguished from further chronic lymphoproliferative disorders according to the scoring system recommended by **Moreau *et al.*** [9].

6- Blood samples were collected during routine follow-up visits to our institution. Five milliliter of venous blood were withdrawn from each patient and divided into three tubes; the first two tubes contained ethylene diamine tetra acetic acid (EDTA as an anticoagulant): one for performing complete blood counts (CBC) and peripheral blood films stained with Leishman's stain to determine differential leukocyte counts.

The second tube was used for immunophenotyping and the third one for serum biochemistry tests.

7- Flow-Cytometric Immunophenotyping:

Flow-cytometric immunophenotyping to define the classical immunophenotyping panel for identification of CLL. It was performed using CLPD panel where panel of monoclonal antibodies was used: (lymphoproliferative diseases panel) CD5, CD19, CD20, CD22, CD23, CD79b, FMC7, CD10, CD38, CD3, CD4,CD8 as well as κ and λ light chains labeled with either fluorescin isothiocyanate (FITC) or phycoerythrin (PE).

Markers were considered positive if ≥ 20% of cells expressed that marker. The whole blood lysis staining method was done. A total of 10,000 events were routinely acquired.

8- Anti-CD38 monoclonal antibody (FITC) was used to analyze CD38 expression, and CD38 expression was assessed as the percentage of CD38-positive cells of the gated B cells (CD19+/CD5+). Patients were considered positive for CD38 when ≥ 30.0% leukemia cells (CD19+/CD5+) expressed it.

9-Human leucocyte antigen-G (soluble form) was defined by assay method [sandwich enzyme-linked immunosorbent assay (ELISA)].

Ethical aspects:

Prior to subject recruitment, the study protocol was reviewed and approved by the Research Ethics Committee of the Faculty of Medicine, Aswan University.

Signed informed consents were obtained from all the participants in the study.

Statistical analysis

Recorded data were analyzed using the statistical package for social sciences, version 20.0 (SPSS Inc., Chicago, Illinois, USA). Quantitative data were expressed as mean ± standard deviation (SD).

Qualitative data were expressed as frequency and percentage. Independent-samples t-test of significance was used when comparing between two means. Chi-square (x^2) test of significance was used in order to compare proportions between two qualitative parameters. The confidence interval was set to 95% and the margin of error accepted was set to 5%.

The p-value was considered significant as the following:

- P-value ≤ 0.05 was considered significant.
- P-value ≤ 0.001 was considered as highly significant.
- P-value > 0.05 was considered insignificant.

RESULTS

The study included two groups. The first group included 30 newly diagnosed patients with CLL (the CLL Group). The second group included 10 healthy volunteers (the Control Group).

Characteristics of the B-CLL Patients

In order to evaluate the prognostic significance of HLA-G in CLL, a cohort of 30 newly diagnosed B-CLL patients, 18 males and 12 females (mean age 58.8 ± 12.1 years). Our patients were classified according to modified Rai staging system **Rai *et al.*** [8] into 2 subgroups:

- Low risk (stage 1 and 2): included (19) patients (63.3%).
- High risk (stage 4) included (11) patients (36.7%).

Table (1): Findings of laboratory tests of the two studied groups

	CLL Group n=30	**Control Group n=10**	**p value**
Hemoglobin (mg/dL)	11.0 ±1.4	12.3 ±1.5	0.03
TLC ($x10^3/mm^3$)	48.5± 11.8	5.8 ± 1.6	<0.001
ALC ($x10^3/mm^3$)	42.2±9.8	2.2±0.5	<0.001
Platelets ($x10^3/mm^3$)	132 ±31.6	235 ±56.3	<0.001

Data are presented as mean ± SD or median (range), TLC: Total leukocytic count, ALC: absolute lymphocyte count.

Table (1) showed that Haemoglobin concentration and platelet count were significantly lower in CLL group. Meanwhile, total leukocytic count and percentage of lymphocytes were significantly higher in CLL group.

Table (2): Correlation between soluble HLA-G forms in CLL group and laboratory parameters (n=30)

		HLA-G Soluble form
Hemoglobin	r	0.155
	p	0.414
TLC	r	0.077
	p	0.684
ALC	r	0.087
	p	0.649
Platelets	r	0.400
	p	0.028
LDH	r	0.133
	p	0.483
β2 microglobulin	r	0.068
	p	0.720

TLC: Total leukocytic count, ALC: absolute lymphocyte count, r = correlation coefficient, p = p value

Table (2) showed that soluble HLA G was positively correlated with the platelets count (r = 0.400, p = 0.02), but not associated with other laboratory parameters.

Table (3): Relation between level of soluble form of HLA-G and age, sex and Rai stage

	HLA-G Soluble form (pg/ml)		**p value**
	≤ 104.80	**> 104.80**	
Age:			
< 60 years	12 (80.0%)	3 (20.0%)	0.001
≥ 60 years	3 (20.0%)	12 (80.0%)	
Gender:			
Male	7 (70.0%)	3 (30.0%)	0.121
Female	8 (40.0%)	12 (60.0%)	
Rai stage:			
Stage 1	4 (57.1%)	3 (42.9%)	0.071
Stage 2	3 (25.0%)	9 (75.0%)	
Stage 4	8 (72.7%)	3 (27.3%)	

Table (3) showed that higher level of soluble form HLA-G was significantly more frequent in patients 60 years or older (p = 0.001), while it was not associated with all other laboratory parameters.

Table (4): Relation between level of soluble form HLA G and laboratory parameters.

		HLA-G Soluble form (pg/ml)		p value
		≤ 104.80	**> 104.80**	
TLC (x10³/mm³)	≤ 50	7 (46.7%)	8 (53.3%)	0.715
	> 50	8 (53.3%)	7 (46.7%)	
ALC (x10³/mm³)	≤ 42.2	7(46.7%)	8(53.3%)	0.5
	> 42.2	8(53.3%)	7(46.7%)	
Hemoglobin (mg/dL)	≤ 10	3 (40.0%)	3 (60.0%)	1
	> 10	12 (60.0%)	12 (40.0%)	
Platelets (x10³/mm³)	≤ 150	11 (61.1%)	7 (38.9%)	0.136
	> 150	4 (33.3%)	8 (66.7%)	
Lactic acid dehydrogenase (U/L)	≤ 280	12 (57.1%)	9 (42.9%)	0.427
	> 280	3 (33.3%)	6 (66.7%)	
β2 microglobulin (µg/ml)	≤ 3	7 (63.6%)	4 (36.4%)	0.256
	> 3	8 (42.1%)	11 (57.9%)	
CD38 expression	Positive	8 (61.5%)	5 (38.5%)	0.269
	Negative	7 (41.2%)	10 (58.8%)	

Data are presented as number (%).

Table (5): Relation between CD 38, soluble HLA G and laboratory parameters

	CD38 +ve	**CD38 –ve**	**p value**
	n=13	**n=17**	
HLA-G Soluble form (pg/ml)	104 ±25	115 ±27.6	0.183
LDH (U/L)	276.7 ± 111.2	241.8 ± 49.4	0.773
β2 microglobulin (µg/ml)	4.5 ± 1.4	3.3 ± 0.7	0.012
HB (g/dl)	10.3 ± 1.8	11.5 ± 0.6	0.008
Platelets (x10³/mm³)	91±21.4	164 ±39.9	0.025

Table (5) showed that positive CD38 expression was significantly associated with lower Hb and platelets (p = 0.008 and 0.025 respectively).

Also with higher Beta2 microglobulin (p = 0.012) but not associated with soluble HLAG or LDH.

Table (6): Overall survival and its relation to the prognostic factors

		N	No of deaths	Cumulative survival at 18 months (%)	p-value
HLA-G Soluble form (pg/ml)	≤ 104.80	15	2	86.70%	0.632
	> 104.80	15	3	80.00%	
ALC (x10³/mm³)	≤ 42.2	15	2	80.00%	0.788
	> 42.2	15	3	85.00%	
Hemoglobin (mg/dL)	≤ 10	6	4	33.30%	<0.001
	> 10	24	1	95.80%	
Platelets (x10³/mm³)	≤ 150	18	5	72.20%	0.052
	> 150	12	0	100.00%	
LDH (U/L)	≤ 280	21	2	90.50%	0.077
	> 280	9	3	66.70%	
β2 microglobulin (µg/ml)	≤ 3	11	0	100.00%	0.071
	> 3	19	5	73.70%	
CD38 expression	Positive	13	5	61.50%	0.005
	Negative	17	0	100.00%	

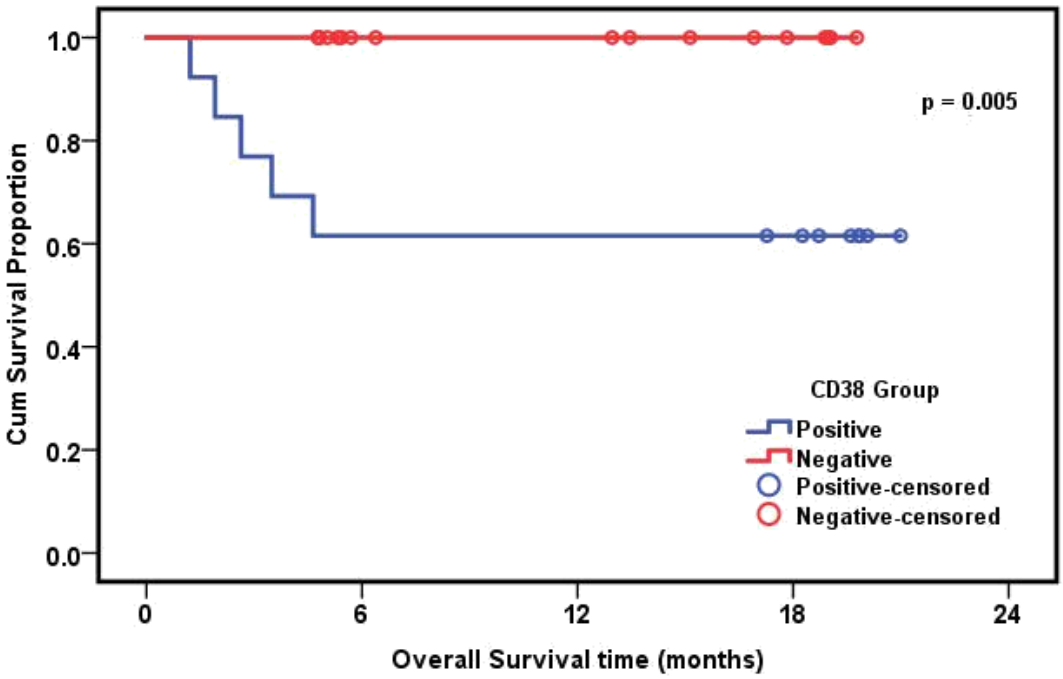

Fig. (1): Overall survival in relation to the expression of CD38

The cumulative overall survival was 83.3%. Positive CD 38 expression was associated with significantly worse survival ($p < 0.005$). HB count < 10 gm/dl was associated with significantly worse survival ($p < 0.001$).

Similarly, patients and those Rai stage 4 had significantly worse survival ($p = 0.005$) (Table 6 and figure 1).

DISCUSSION

There is inadequate documents concerning the frequency of CD38 positivity or its clinical use and relationship with overall survival in Egyptian CLL patients. We carried out this study to estimate the level of CD38 expression in CLL patients, to find its association with baseline clinical and laboratory parameters at presentation and to determine its prognostic value and relation with overall survival.

In our study, positive CD38 expression was significantly associated with lower Hb and platelets ($p = 0.008$ and 0.025 respectively), also with higher Beta2 microglobulin ($p = 0.012$). Similar results were obtained by **Assem *et al.*** [10], the expression of CD38 by CLL cells is associated with an aggressive clinical presentation that is confirmed by many studies [11].

The cumulative overall survival was 83.3%. Positive CD 38 expression was associated with significantly worse survival ($p < 0.005$). HB count < 10 gm/dl was associated with significantly worse survival ($p < 0.001$). Similarly, patients and those Rai stage 4 had significantly worse survival ($p = 0.005$). This is in agreement with **He *et al.*** [12]. While **Assem *et al.*** [10] found inverse relation between CD38 expression and time to disease progress ($p = 0.033$), while no significant relation was encountered with overall survival ($p = 0.197$) in Egyptian CLL patients.

The progression-free intervals in patients who are CD38 +ve are shorter, and they die earlier when compared to the CD38 −ve patients [4]. This observation stimulated many clinical centers to accept the purpose of CD38 percentage expression as part of the regular investigations of CLL patients [13].

Flowcytometry is a valuable instrument for population analysis. Its significance is in allowing measurements of multiple features in one cell. Light scattering can detect changes in size and internal complexity and the fluorescence released from labeled antibodies can detect cell surface and intracellular antigens [14].

The study of both CD38 and ZAP-70 would afford respected data in the diagnostic work-up of B-CLL patients [12]. CD38 expression similarly associates with the IgVH mutation status [15, 16].

HLA G, is non-conventional MHC molecule having well tolerating properties, initially was expressed on trophoblast cells to defend fetus from attack by mother immune system [17]. HLA-G can be expressed as seven different isoforms, including four membrane bound (HLA-G1 to -G4) and three soluble (HLA-G5 to - G7) molecules [18]. HLA-G is expressed not only on the cell surface, but also in body fluids. Soluble HLA-G has been found consequently in serum, in amniotic fluid and in CSF. Whether it is also present in urine, tears and milk. Soluble HLA-G molecules may also be shed via the proteolytic cleavage of membrane-bound HLA-G1 [19].

HLA G is measured as an immuoregulatory molecule. HLA G antigens are doing as ligands for immunoglobulin-like transcript 2 (ILT2) and immunoglobulin-like transcript 4 (ILT4), which are expressed by B cells/T cells/NK cells/monocyte, therefore inhibiting proliferation, cytotoxicity of NK cells and reducing T cell task. So, inducing immunosuppression [20].

Preceding studies showed that membrane-bound and soluble HLA-G isoforms expression were associated with various immunosuppressive functions during the progress of malignancies. This was produced with multiple mechanisms such as hindering immune cell function, encouraging apoptosis and the induction of regulatory cells through receptor binding and/or trogocytosis, and weakening chemotaxis of different immune effector cells [18, 19].

Also, increased HLA-G expression has been found in several malignancies, viral diseases, inflammatory, autoimmune disorders and transplantation [21]. The generation of immune tolerance by HLA G is the base used by tumor cells to escape host immune response [22].

CONCLUSION

CD38 expressions are considered potent prognostic markers in predicting overall survival for CLL patients in Egypt and they could be measured for the choice of patient's therapy and to define disease prognosis.

These markers should also be confirmed in a large-scale study to determine their prospective in stopping recurrent relapses and progress of resistance to chemotherapy in CLL.

Expression of soluble form of HLA-G in CLL patients by ELISA get no extra prognostic importance.

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