Determination of HLA-G Expression and Evaluation of Its Role as a Prognostic Factor in Chronic Lymphocytic Leukemia

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Background: In recent years, the clinical and biological features governing the clinical course of chronic lymphocytic leukemia (CLL) have been most extensively studied. Human leukocyte antigen-G (HLA-G) allows tumor cells to escape from the antitumor effect of the immune system. Recent studies have shown that various tumor cells show an increased HLA-G expression. Data regarding HLA-G expression in CLL are limited and controversial. The aim of this work is to evaluate flow cytometry study of HLA-G expression on cell surface and assess its relationship with other prognostic factors (CD38, ZAP70, beta 2 microglobulin [β 2MG]) in patients with CLL. **Design and methods:** Forty-five newly diagnosed CLL cases. White blood cell count, lymphocyte absolute count, hemoglobin level, platelet count, serum lactate dehydrogenase activ-

ity, and serum β 2MG level were studied at admission. In each patient, morphologic diagnosis of B-CLL was confirmed by flow cytometry HLA-G, CD38 and ZAP70 expression levels were measured with four-color flow cytometry. **Results:** HLA-G positivity ranged between 1% and 12% in CLL patients. A significant correlation was found with CD38, ZAP70, disease stage, and β 2MG ($P < 0.001$). The off-treatment follow-up period was longer in the HLA-G negative group ($P < 0.022$). **Conclusions:** In conclusion, we suggest that, in addition to other prognostic factors, surface HLA-G expression can be considered as an independent prognostic factor. However, our work should be confirmed by further prospective studies, a longer off-treatment follow-up period, and a standardized method. *J. Clin. Lab. Anal.* 30: 399–403, 2016. © 2015 Wiley Periodicals, Inc.

Key words: chronic lymphocytic leukemia (CLL); human leukocyte antigen-G (HLA-G); prognostic factors

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a common type of leukemia in adults. Its clinical course is extremely heterogeneous, with some patients surviving without the need for a specific treatment while some others need an urgent therapy. Currently, the methods defining the clinical and biological features of the disease that determine its clinical course are the subject of many works (1). There are two current staging systems for CLL (RAI and BINET). There is a significant correlation between the disease stage and survival, although this relationship has also some scientific gaps. Disease stage cannot differentiate progressive disease from a more stable disease course (2, 3). Thus, various parameters determining prognosis independently of the disease stage have been developed. Various prognostic factors have been defined for CLL including lactate dehydrogenase (LDH), beta 2 microglobulin (β 2MG), thymidine kinase, serological markers such as sCD23, lymphocyte doubling time, bone marrow infiltration type,

cytogenetic abnormalities, CD38 and ZAP70 expression, and the state of the mutation at the variable zone of the immunoglobulin heavy chain gene (Ig VH) (2, 4–6).

One of the novel parameters relevant to the prognosis of CLL is the human leukocyte antigen-G (HLA-G) positivity. HLA-G is a nonclassical HLA class I antigen (7). It does not exist in normal tissues. It is found in trophoblasts of early gestational placenta (8). The HLA-G gene is found in peripheral blood, B and T lymphocytes, and monocytes. It has seven different isoforms, four of which code for membrane-bound proteins and three code for free proteins (9). HLA-G has many functions related to immunoregulation. By binding to CD8, it behaves as

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a peptide-presenting molecule and mediates immune tolerance. It directly or indirectly protects target cells from NK cytotoxicity via many inhibitor receptors (P49, ILT-2, KIR) expressed on NK cells (10). It prevents antigen-free lysis via surface molecules expressed on cytotoxic CD8 T cells and thus causes tumor progression by enabling tumors to escape from immune system (7, 10). Recent studies have shown that various tumor types (B- and T-cell lymphomas, lung cancer, colon cancer, melanoma, breast cancer, renal cancer, and gliomas) exhibit increased HLA-G expression (10–12). HLA-G expression is detected either on cell surface or, in case of the free forms, in serum (13). Although many studies have shown an increase in the circulating form of HLA-G in CLL, its expression on cell surface and prognostic importance are controversial (7, 14–18).

The aim of this work is to evaluate four-color flow cytometry study of HLA-G expression on cell surface and assess its relationship with other prognostic factors (CD38, ZAP70, β 2MG) in patients with CLL.

MATERIALS AND METHOD

Patients

This study retrospectively examined medical records of 45 patients who were diagnosed with CLL according to National Cancer Institute-sponsored Working Group (NCI-WG) guidelines for CLL (19) between October 2009 and July 2011 at Ankara Numune Training and Research Hospital, Department of Hematology. The study was approved by the Ankara Numune Training and Research Hospital Ethical Committee.

Twenty-nine patients were male and 16 were female. The mean age of the study population was 68.7 ± 8.2 years (see Table 1).

White blood cell count, lymphocyte absolute count, hemoglobin level, platelet count, serum LDH activity, and serum β 2MG level were studied at admission. The clinical stage was determined according to the RAI staging system (20). In each patient, morphologic diagnosis of B-CLL was confirmed by flow cytometry, CD5⁺ CD19⁺ CD20⁺ CD23⁺ Ig light chain κ or λ immunophenotype. Time from diagnosis to the initial treatment was calculated for all patients. HLA-G expression was determined from blood samples drawn from 20 healthy donors.

Flow Cytometry

HLA-G, CD38, and ZAP70 expression levels were measured with four-color flow cytometry. The monoclonal antibodies (MoAbs) used for tagging in flow cytometry were obtained from Beckman Coulter (BC) company. CD45 Fluorescein Isothiocyanate (FITC), CD79b Phycoerythrin (PE), CD11c Phycoerythrin-Cyanin 5

TABLE 1. Demographic Features

Variables	n = 45
Age (years)	68.7 $\pm$ 8.2
Sex	
Female (%)	16 (35.6)
Male (%)	29 (64.4)
White blood cell/ μ l	30 (11.9–298)
Lymphocyte/ μ l	25 (70–280)
Hemoglobin, g/dl	12.8 $\pm$ 1.58
PLT/ μ l	200 (18–396)
β 2 microglobulin, mg/l	3.6 (2.0–9.2)
HLA-G %	10 (0–12)
CD38 %	10 (0–81)
ZAP70 %	12 (1–78)
Stage RAI	
Stage 0	21 (46.7%)
Stage 1	6 (13.3%)
Stage 2	4 (8.9%)
Stage 3	10 (22.2%)
Stage 4	4 (8.9%)
Off-treatment follow-up period (months)	10 (0–24)

(PC5), CD19 Phycoerythrin-Texas Red (ECD), CD5 FITC, CD10 PE, CD14 PC5, CD20 ECD, CD22 PE, CD23 PE, CD38 PC5, CD103 FITC, CD43 PE, Kappa FITC, Lambda PE, ZAP70 FITC, and CD2 PC5 MoAb were used for this purpose. A specific MoAb, MEM/G9 FITC (Novus Biologicals), was used for HLA-G. IgG1 FITC, IgG1 PE, IgG2a ECD, and IgG1PC5 MoAb were used as isotypic control.

Peripheral blood samples were drawn into K3 EDTA containing tubes for flow cytometric examination. Ficoll-Paque density gradient centrifugation was used to separate mononuclear cells in peripheral blood from freshly drawn anticoagulated blood. In order to block the non-specific binding, the cells were preincubated for 5 min with mouse serum prior to staining. Direct immunofluorescence technique was employed for surface staining with a standard four-color flow cytometry approach. One hundred microliters (1×10^6 cells) peripheral blood mononuclear cells were incubated in 200 μ l PBS at room temperature for 30 min in the dark with 10 μ l MoAb. After two washing steps with Phosphate-Buffered Saline (PBS) solution. Acquisition and analysis of the cells were performed in the FC 500 (BC) flow cytometry device using CXP program. All samples possessed an immunophenotype of CD5⁺ CD19⁺ CD20⁺ CD23⁺ Ig light chain κ or λ . Analysis of CD38 expression was performed by gating from CD5⁺ CD19⁺ cell population. HLA-G expression was evaluated in CD5⁺ CD19⁺ cell population after its expression on T cells and monocytes was excluded. A commercially available fixation/permeabilization kit was used to determine intracellular ZAP70 expression. Briefly, 1×10^6 Mononuclear Cells (MNCs) were first surface stained

Fig. 1. (A) Analysis of Gate A from FS/SS lymphocytes, (B) HLA-G on CD5+19+ cells, (C) CD38 expression, (D) ZAP70 expression.

with CD2/CD56/CD19 and then fixed, permeabilized, and stained with ZAP70 FITC MoAb. Its expression on ZAP70, T, and NK cells was excluded and evaluated in CD19⁺ B cells. A cut-off level of 20% was taken for CD38 and 20% for ZAP70. HLA-G expression ranged between 0% and 12% (Fig. 1).

Statistical Analysis

Statistical Package for Social Sciences (SPSS) version 11.5 software (SPSS Inc., Chicago, IL) was used for data analysis. The normality of distribution of the study data was tested using Shapiro–Wilk test. Depending on the normality of distribution, mean $\pm$ standard deviation or median (minimum-maximum) value was used to express continuous variables. Categorical data were presented as the number of cases and percentages. An Receiver Operating Characteristic (ROC) curve was drawn to calculate the area under curve and 95% confidence intervals for CD38 and HLA-G levels for discrimination of RAI stage groups. For HLA-G, sensitivity, specificity, and positive and negative predictive values were calculated for various cut-off points to determine their best. A *P* value less than 0.05 was considered statistically significant.

RESULTS

This study included a total of 45 patients (29 males, 16 females; mean age 68.7 ± 8.2 years) with CLL.

TABLE 2. Sensitivity, Specificity, and Positive and Negative Predictive Values of HLA-G at the Best Cut-Off Points for Differentiation of Stages 3 and 4 From Stages 0, 1, and 2

Variables	Sensitivity	Specificity	PPV	NPV
Stages 0, 1, and 2 vs. Stages 3 and 4				
≥ 5	85.7%	71.0%	57.1%	91.7%

PPV, positive predictive value; NPV, negative predictive value.

Various clinical and biological variables including age at diagnosis, sex, lymphocytosis, platelets, hemoglobin, LDH, β 2MG, RAI stage at diagnosis, and time from diagnosis to the first treatment were retrospectively analyzed. The mean off-treatment follow-up period was 10 (0–24) months. HLA-G expression on CLL cells ranged between 0% and 12% and was higher than that of the control group (0–0.03%). By using an ROC curve, the cut-off level of 5% was determined for HLA-G, above which all values were considered positive (Fig. 2). The positive and negative predictive values to differentiate Stages 0, 1, and 2 from Stages 3 and 4 were 57% and 91.7%, respectively (Table 2). Except for two patients, all patients in the HLA-G positive group ($n = 10$) needed treatment at admission. In the HLA-G negative group ($n = 35$), on the other hand, three patients received chemotherapy in the first year. Thirty-two patients were followed without any treatment and three patients were lost to follow-up during off-treatment period. Spearman's correlation analysis

Fig. 2. ROC curve of HLA-G for differentiation of Stages 3 and 4 from Stages 0, 1, and 2.

TABLE 3. Correlation Coefficients and Their Significance Between HLA-G and CD38, ZAP70, Stage RAI, and β 2 Microglobulin

Variables	Correlation coefficient	<i>P</i> value
CD38	0.647	<0.001
ZAP70	0.648	<0.001
Stage RAI	0.541	<0.001
β 2 microglobulin	0.668	<0.001

showed that there was a significant inverse (negative) correlation between the off-treatment follow-up period and HLA-G level ($r = -0.357$ and $P = 0.022$). HLA-G expression was significantly correlated with certain prognostic factors, namely CD38, ZAP70, disease stage, and β 2MG ($P < 0.001$; Table 3).

DISCUSSION AND CONCLUSION

Many studies shedding light on CLL biology have been conducted in the last 10–15 years. They have particularly clarified the pathogenesis of the disease and discovered novel parameters that are reportedly effective on prognosis.

HLA-G enables tumor cells to escape from the antitumor action of immune system. Recent studies have shown that various tumor cell types (B- and T-cell lymphomas, lung cancer, colon cancer, melanoma, breast cancer, renal

cancer, and glioma) are characterized by increased levels of HLA-G expression (10–12).

There are limited and controversial data on HLA-G in CLL. HLA-G was first studied in hematological malignancies by Mizuno et al. (15). They explored HLA-G in 40 subjects with acute leukemia and found it negative in all samples. Polakova et al. found negative HLA-G expression with flow cytometry in 51 patients with leukemia (16). However, these studies used the indirect method and 87G antibody as the MoAb for detection of HLA-G expression in flow cytometry. Nuchel et al. retrospectively assessed 47 patients with CLL. They made a positive immunomagnetic selection by using anti-CD19 microbead from peripheral blood mononuclear cells with the direct method and employed human serum to prevent nonspecific binding. They detected HLA-G by making paired staining with MEM/G9 MoAb and found varied levels of HLA-G positivity (1–54%) in all samples (21). They observed a more aggressive course in patients with HLA-G positivity exceeding 23%. They reported that, as compared to other prognostic factors (disease stage, β 2MG, CD38, ZAP70), HLA-G expression could be used as an independent predictor for disease progression. Unlike the study of Nuckel et al., Perez-Chacon et al. achieved to obtain a pure cell population over 95% by also employing a negative selection with anti-CD2 and anti-CD14 immunomagnetic beads in a larger study containing 169 patients and 20 healthy controls. They found a low rate of HLA-G positivity (0–14.5%; as opposed to HLA-G positivity of 0.2–0.4% in healthy controls) in an analysis made by excluding dead cells and cell aggregates from CD5+CD19+ cells again using MEM/G9 MoAb. In contrast, they found a higher anti-HLA-G positivity rate in an analysis without excluding cell aggregates (0.1–45%). Giannopoulos et al. detected an HLA-G positivity rate of 0.3–60.3% without using MEM/G9 MoAb and anti-CD19 microbead in 20 B-CLL patients and found no significant relationship between HLA-G positivity and disease stage, CD38, and ZAP70 (22). In a 20-patient study, Erikçi et al. found an HLA-G positivity rate in the range of 1–34% using the direct method employing whole blood lysis with MEM/G9 MoAb without anti-CD19 microbead and human serum for blockade. They found a relationship between HLA-G and disease stage, disease-free survival, but not with CD38 and ZAP70 and attributed this finding to the small sample size (17). Lastly, Attia et al. found an HLA-G positivity rate of $35.31 \pm 12.35\%$ and reported that there was a significant correlation between HLA-G positivity and CD38 in 30 CLL patients. They suggested that HLA-G may be a useful marker for prediction of disease progression (18). As is understood from all the above-mentioned studies, there are some contradictory points with respect to both expression and study techniques of HLA-G. We found a low rate

of HLA-G expression (0–12%). Methodologically, unlike Nuckel et al., we used no CD19 microbead and excluded HLA-G expression in T lymphocytes and monocytes as well as debris and cellular aggregates at the analytic stage, but other steps we used were almost identical with those followed by Nuckel et al. However, we found a lower rate of HLA-G expression as Perez-Chacon et al. did. That much variation in different studies is a result of nonstandardized study methods. We believe that it is a priority to standardize the study method and determine the cut-off level before starting to study HLA-G expression in such patients.

Nuckel et al. suggested to use HLA-G as an independent predictor of disease progression while Attia et al. reported that HLA-G was correlated to other prognostic factors and may be an important prognostic parameter for disease progression. In contrast, Perez-Chacon et al. and Giannopoulos et al. found no significant correlation with disease stage, CD38, and ZAP70. Erikçi et al. found a relationship between HLA-G and disease stage but not with CD38 and ZAP70. In our study, we found a positive predictive value of 57% and a negative predictive value of 91.7% for differentiation of Stages 0, 1, and 2 from Stages 3 and 4. There was a significant correlation between disease stage and HLA-G (Table 2).

Limitation of this study is unutilization of CD19 microbead and the shorter duration of untreated follow-up period. The off-treatment follow-up period was short in our study but we still analyzed its correlation with HLA-G. There was a significant inverse (negative) correlation between the follow-up period and HLA-G level ($r = -0.357$ and $P = 0.022$).

There was a significant correlation with other prognostic factors, namely CD38, ZAP70, disease stage, and $\beta 2\text{MG}$ (Table 3; $P < 0.001$). Similar to Nuckel et al. and Attia et al., more than 35 prognostic factors are defined in CLL. Although several laboratory tests such as IGHV mutation, cytogenetic investigation cannot be performed in every laboratory due to their higher costs, flow cytometric methods are relatively cheaper and easier. Once the optimum cut-off level and standardized method for HLA-G expression by flow cytometry are determined, we are of the opinion that HLA-G can be used as a prognostic factor; however, our results should be confirmed by prospective studies with a longer follow-up period. All the above-mentioned studies collectively suggest that defining a standardized method for HLA-G expression should be primarily pursued.

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