## **Short Report**

# Analysis of HLA-G Gene Expression in B-Lymphocytes from Chronic Lymphocytic Leukemia Patients

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Received 3 April 2006; revised 18 October 2006; accepted 22 October 2006

## ABSTRACT

**Background:** The human leukocyte antigen G (HLA-G) molecule exhibits limited tissue distribution, low polymorphism and alternative splicings that generate seven HLA-G isoforms. HLA-G exerts multiple immunoregulatory functions. Recent studies indicate an ectopic up-regulation in tumor cells that may favor their escape from anti-tumor immune responses. This study it is an effort to clarify the presence of HLA-G in B-cell chronic lymphocytic leukemia (B-CLL) patients. **Methods:** HLA-G mRNA expression was studied in a pilot study in circulating B-CLL and also healthy controls by reverse transcription (RT)-PCR using a set of pan-HLA-G primers. **Results:** RT-PCR was performed on B-cells from 74 B-CLL patients and 12 healthy controls. The data showed HLA-G gene expression in 20% of the B-CLL patients. No expression of HLA-G could be detected in the healthy control group. **Conclusion:** These data suggest that HLA-G is expressed at the gene level in B cells from B-CLL patients but not in B cells from healthy controls. Further study is required to clarify the role of HLA-G as a regulatory factor that could affect immune response in B-CLL patients. *Iran. Biomed. J. 11 (2): 125-129, 2007* 

Keywords: Human leukocyte antigen G (HLA-G), Reverse transcription PCR (RT)-PCR, B-cell chronic lymphocytic leukemia (B-CLL)

## **INTRODUCTION**

cell chronic lymphocytic leukemia (B-CLL) is the most common adult leukemia in western countries. It is characterized by an accumulation of long lived antigen experienced B lymphocytes that differ in the level of immunoglobuline V-gene mutation [1]. B-CLL has a variable clinical course. Some patients have good prognosis and never require treatment, whereas in others survival is short despite early initiation of therapy. During the last few years, there has been a continuous effort to identify novel prognostic factors in B-CLL that may help to define patient subgroups could benefit from early therapeutic that intervention. Human leukocyte antigen G (HLA-G) expression could be used to predict the prognosis of disease. HLA-G is a non-classical major histocompatibility complex class I antigen with very little sequence variability and alternative splicings of its primary transcript that lead to four membrane-bound isoforms and three soluble isoforms. It is not expressed in normal tissues except in throphoblast from early gestation placentas [2], thymic epithelial cells and cornea [3]. HLA-G exerts multiple immuno-regulatory functions such as inhibition of natural killer (NK) or T-cell apoptosis, or inhibition of transendothelial NK cell migration [4] through three inhibitory receptors: ILT2 (NK cells, T cells, APC), ILT4 (myeloid cells) and KIR2DL4 (NK cells and T cells), the latter being a specific HLA-G receptor [5]. Since the net results of these effects are immuno-suppression, HLA-G expression in tumor cells may favor their escape from anti-tumor immune responses, thus allowing tumor progression [6]. HLA-G co-expressed in the presence of classical HLA class I molecules may also play an important immuno-modulatory role in tumor cells [7]. HLA-G expression has been yet demonstrated on certain types of tumors such as melanoma [8, 9] renal [10], breast [11], lung [12] carcinoma, glioma [7], cutaneous lymphoma [12], B-cell and T-cell lymphomas [13], malignant ascits [14] and gastrointestinal cancer [15]. The present study aims to analyze HLA-G gene expression in B-CLL patients in order to gain insight into the role of HLA-G in this disease.

## MATERIALS AND METHODS

**Patients and controls.** B-CLL patients (n = 74) with a mean age of 67 years were included in the study. The bleeding was conducted at different stages of the disease. Healthy controls (n = 12) were also included in the study.

*Isolation of blood mononuclear cells.* Peripheral blood was collected in sterile heparin zed tubes. Peripheral blood mononuclear cells were isolated by Ficoll-Paque (GE Health care, Uppsala, Sweden) gradient centrifugation at most 6-8 hours after samples collection [16].

*Cell line.* JEG-3 (Human placental choriocarcinoma cell line) cell line [2] that consistently expresses mRNA (a kind gift from Professor Håkan Mellstedt, Karolinska Institute, Stockholm, Sweden) was used as positive control.

**RNA extraction and cDNA synthesis.** Total RNA was extracted from  $1-2 \times 10^6$  lymphocytes, using RNA-Bee (BioSite, Stockholm, Sweden) based on the guanidium thiocyanate phenol-chloroform extraction method [17, 18].

**Reverse transcription (RT)-PCR analysis.** Specific amplification of HLA-G transcripts was performed on cDNA. First strand cDNA synthesis was performed as described earlier [18]. PCR was performed in total volume of 25  $\mu$ l containing 1  $\mu$ l of cDNA, 0.5 U of Red hot® DNA polymerase enzyme (AB gene, U.K), 200 mM of dNTPs (Promega, Falkenberg, Sweden), 10 pmol of each

primers, 1.5 mM Mgcl<sub>2</sub>. The following sets of HLA-G primers were used: Forward [5'-GGAAGAGGA GACACGGAACA-3'] and Reverse [5'-CCTTTTCA ATCTGAGCTCTTCTTT-3']. As an internal control, the β-Actin gene amplification was carried out under the same PCR conditions for each sample using the following set of primers: forward [5'-ATGTTTGAGACCTTCAACAC-3'] and Reverse [5'-CACGTCACACATGATGG-3']. The PCR cycle conditions were 3 min at 95°C, 45 s at 95°C, 30 s at 60°C, 30 s at 72°C for 35 cycles with the final extension at 72°C for 7 min. PCR products were analyzed by gel electrophoresis on 1.5 % ethidium bromide stained agarose gel.

### RESULTS

**HLA-G gene expression.** HLA-G gene expression was different in male and female patients. It showed more frequency of expression in female patients (60%) rather to male (40%) as shown in Figure 1. Median age of patients was 66 years with high prevalence in 74-83 years. Percentage of female and male patients was 63.5% and 36.5%, respectively.

Amplification of HLA-G transcripts of JEG-3 cells, revealed three bands corresponding to HLA-G1 (730bp), HLA-G2/G4 (450bp) and HLA-G3 (220bp). Similarly, HLA-G gene expression was detected in 14 of 74 patients (19%) as shown in Figures 2 and 3. No HLA-G gene expression was detected in cells isolated from normal controls (data not shown). All samples from B-CLL patients and healthy controls were positive for actin gene expression.



**Fig. 1.** The percentage of HLA-G expression in male and female B-CLL patients.



**Fig. 2**. Percentage of B-CLL patients which express HLA-G on leukemic B cells.

#### DISCUSSION

It has been suggested that HLA-G antigens contribute to immune evasion of tumor cells [19], and HLA-G transcriptional activity has been observed in many tumor tissues and cell lines. However, cell surface HLA-G antigens have only been found in a few samples tested [13, 20]. It is suggested that HLA-G expression is regulated at transcriptional and post transcriptional levels. The factors involved in this regulation remain to be identified [13, 20]. Data regarding HLA-G expression in B-CLL is limited and controversial. While Amiot *et al.* [21] found transcription of the HLA-G gene in 3 of 6 B-CLL patients using RT-PCR, Mizuno *et al.* [22] were unable to detect the HLA-G antigen by flow cytometry in B-CLL patients even after activation of B-cells with IFN- $\gamma$ . Polakova *et al.* [20] showed HLA-G antigen is not expressed in freshly isolated human leukemia cells in B-CLL patients and therefore suggested it may not be involved in their escape from immune attack.

Nuckel *et al.* [23] were able to demonstrate HLA-G antigen expression in a variable proportion of B-CLL leukemic cells. It was shown that the proportion of leukemic cells expressing HLA-G varied from 1% to 54%. Patients with 23% or fewer HLA-G-positive cells had a significantly longer progression-free survival time than patients with more than 23% positive cells [23]. Furthermore, humoral and cellular immuno-suppression were significantly more prominent in the HLA-G-positive patient, as compared with that of HLA-G-negative patient group [23].



**Fig. 3.** Electrophoretic gel with PCR product of actin and HLA-G shown in B-CLL patients. NC, negative control; PC, positive control; JEG-3 cell line, MW, molecular weight marker (100 bp). Representative gel of PCR products in B-CLL patients.

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study, HLA-G expression In this gene demonstrated in 19% of B-CLL patients but not in healthy controls. The result of this study is consistent with other studies [22, 23]. Since, HLA-G expression may contribute to the impairment of immune responses against tumor cells in B-CLL patients; it seems the level of HLA-G expression is correlated with the degree of immunosuppression and stage of the disease. Thus, this study needs to be expanded to a larger group of progressive and nonprogressive B-CLL patients.

The absence of HLA-G in healthy controls is most probably due to lack of HLA-G gene expression and an approach with a large number of healthy controls using pure CD19 B- cells is warranted to clarify the matter. Detection of HLA-G antigen expression at protein level apart from problems related to small numbers of HLA-G molecules on the cell surface might be due to different stages of the disease. In addition, controversial results on different studies are likely to be explained by differences in the properties of the monoclonal antibodies used to detect HLA-G antigen. For example it was shown that direct immunofluorescence using MEM/G9 antibody may be a more sensitive approach to visualize the HLA-G antigen [22]. Furthermore, it is advisable to measure HLA-G expression in CD19enriched B-CLL cells [23]. Considering the fluctuations in HLA-G detection with flow cytometry, real time quantitative RT-PCR will be a useful method to precisely detect the level of HLA-g transcripts in B-CLL. Furthermore, factors like cytokines that may have role/roles on HLA-G expression could be considered in the future studies.

Based on this data and others [22, 23] it may be worth to analyses HLA-G expression (at gene or protein level) in B-CLL patients to predict the outcome of the disease. This method may be more feasible than detection of Zap-70, CD38 receptor or considering mutated and non-mutated CDR3 region of VH genes to predict the prognosis of B-CL patients. Soluble form of HLA-G (sHLA-G) is another factor to be considered in prognosis of the disease. Since, Amiot et al. confirmed that sHLA-G increased in lymphoproliferative disorders [13]. Increased level of sHLA-G may have negative immunomodulatory effects similar to that of membrane bound isoforms in favoring escape of leukemic cells from immune response. These studies including the present data might provide a clinical basis for using HLA-G modulation, as a relevant goal in lymphoproliferative disorders.

It seems that in B-CLL, the level of HLA-G expression correlates with the degree of immunosuppression and prognosis. HLA-G may contribute to the impairment of immune responses against tumor cells and infections. Thus, HLA-G as a regulatory marker may be considered in any protocol for immunotherapy (vaccination) in B-CLL patients. Taken together, these data suggest that HLA-G expression might be a disturbing regulatory factor that could affect immune response in B-CLL patients.

#### ACKNOWLEDGMENTS

The authors would like to express their deep gratitude to Dr. M. Jeddi-Tehrani for his creative comments and corrections.

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