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ORIGINAL ARTICLE

HLA-G and MHC Class II Protein Expression in Diffuse Large B-Cell Lymphoma

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Abstract The expression of human leukocyte antigen-G (HLA-G) and HLA class II protein was studied by immunohistochemical staining of lymph nodes from 148 patients with diffuse large B-cell lymphoma (DLBCL) and related to the clinical course of the disease. Negative HLA-G expression was associated with a lower probability of achieving a complete remission ($p = 0.04$). Patients with negative HLA-G expression tended towards a lower 3-year overall survival (OS) rate compared to those with positive expression of HLA-G ($p = 0.08$). When restricting the analysis to patients receiving chemotherapy with rituximab, the estimated 3-year OS rate of patients with positive HLA-G expression was 73.3 % compared with 47.5 % ($p = 0.03$) in those with negative expression. Patients with negative HLA class II expression presented a lower 3-year OS rate compared to subjects with positive expression ($p = 0.04$). The loss of HLA class II expression ($p = 0.05$) and belonging to the intermediate high/high IPI risk group ($p = 0.001$) independently increased the risk of death. HLA class II expression also retained its prognostic value in patients receiving rituximab; the 3-year OS rate was 65.3 % in patients with positive HLA class II expression versus 29.6 % ($p = 0.04$) in subjects that had loss of HLA class II expression. To our knowledge, for the first time, the expression of HLA-G protein in DLBCL and its association with the clinical course of the disease was demonstrated. Moreover, the link between losing HLA class II protein expression and poor survival of patients treated with immunochemotherapy was confirmed.

Keywords Human leukocyte antigen-G · Major histocompatibility class II · Immunohistochemistry · Diffuse large B-cell lymphoma · Outcome

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Introduction

The clinical course of patients with diffuse large B-cell lymphoma (DLBCL), the most common and heterogeneous subtype of non-Hodgkin lymphoma (NHL), is variable (Swerdlow et al. 2008). It is well known that immune alteration is a major risk factor for developing NHL; however, the specific immune mechanisms that could be responsible for this phenomenon remain unresolved. There is mounting evidence that variations in the genes of the immune system that encode key cytokines for the inflammatory response and T-helper (Th)1/Th2 balance, as well as major histocompatibility complex (MHC) class I and class II and their subsequent altered expression, play a role in the etiology and clinical course of NHL (Bielska et al. 2015; Juszczynski et al. 2002; Lech-Maranda et al. 2004,

2010; Lu et al. 2011; Rothman et al. 2006; Wang et al. 2010; Warzocha et al. 1998).

Human leukocyte antigen-G (HLA-G), a non-classical MHC class I molecule, affects human immunity by inhibiting the activity of natural killer (NK) cells, cytotoxic T lymphocytes and antigen-presenting cells (APCs), which are the main cells involved in the development of a cytotoxic antitumor immune response (Carosella et al. 2008; LeMaoult et al. 2003). HLA-G exerts its inhibitory activity through direct binding to specific inhibitory receptors: immunoglobulin-like transcript LILRB1 (leukocyte immunoglobulin-like receptor, subfamily B, member 1; ILT-2; CD85J) which is expressed by both lymphoid and myeloid cells, LILRB2 (leukocyte immunoglobulin-like receptor, subfamily B, member 2; ILT-4; CD85D) expressed only by myeloid cells, and KIR2DL4 (killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, member 4; CD158D) detected only on NK cells (Carosella et al. 2008; LeMaoult et al. 2003; Loustau et al. 2013).

The expression of HLA-G proteins is highly tissue restricted and physiologically occurs in cytotrophoblast cells as well as in adult thymic medulla, cornea, nail matrix, pancreatic islets, erythroid and endothelial precursors and mesenchymal stem cells (Carosella et al. 2008; Favier et al. 2007). However, the *HLA-G* gene is transcribed in a variety of human adult tissue, peripheral B and T lymphocytes, and monocytes, but not in NK cells (Amiot et al. 1996). The expression of HLA-G proteins has been also observed after organ transplantation and in many pathological conditions such as autoimmune and allergy diseases, viral infections and especially in cancers (LeMaoult et al. 2003; Loustau et al. 2013). The clinical significance of HLA-G expression in solid tumors has been extensively investigated and reported to contribute to cancer progression (Carosella et al. 2008; Favier et al. 2007; Loustau et al. 2013); however, little is known about HLA-G expression and its clinical relevance to hematologic malignancies (Yan 2010). Although in some studies increased expression of HLA-G has been observed in patients with chronic lymphocytic leukemia (CLL) and correlated with adverse clinical outcome (Nuckel et al. 2005; Rebmann et al. 2007; Rizzo et al. 2014), other authors have not shown any association between elevated soluble HLA-G (sHLA-G) plasma levels and the clinical course of lymphoid malignancies (Giannopoulos et al. 2008; Sebti et al. 2007; Park et al. 2008).

MHC class II molecules (HLA-DP, -DQ, -DR) are mainly expressed by professional APCs including B lymphocytes, dendritic cells, and monocytes/macrophages. These proteins are critical for the protective immune response to pathogens and tumors. The role of MHC class II genes in the pathogenesis and prognosis of lymphoid malignancies, especially DLBCL, seems to be well understood (Lu et al. 2011; Rimsza et al. 2004, 2006, 2007; Roberts et al. 2006; Schmelz et al. 2012; Wilkinson et al. 2012;). It has been reported that decreased MHC class II gene expression is accompanied by significantly shortened survival and a decreased percentage of $CD8^+$ tumor-infiltrating lymphocytes (Rimsza et al. 2004). Loss of MHC class II expression in DLBCL is therefore hypothesized to deteriorate tumor immunosurveillance.

The aim of this study was therefore to investigate the expression of HLA-G and MHC class II protein in DLBCL and to relate their expression to the clinical course of the disease.

Materials and Methods

Subjects

The study group consisted of 148 patients with de novo DLBCL diagnosed and treated in the Regional Oncology Center in Lodz, the Department of Hematology at the Medical University of Lodz, and the Institute of Hematology and Transfusion Medicine in Warsaw between 2005 and 2012. The initial medical evaluation consisted of a complete history and physical examination, bone marrow biopsy, computed tomography of the chest, abdomen and pelvis, and blood morphology and chemistry. Patients were graded according to the Ann Arbor classification, and performance status was assessed using criteria from the Eastern Cooperative Oncology Group (ECOG). The International Prognostic Index (IPI) system was used to divide patients into low/intermediate low-risk (IPI score: 0–2) and intermediate high/high-risk (IPI score: 3–5) groups.

Diagnostic samples of lymph nodes from the study patients were reviewed by two expert hematopathologists. Histopathological analysis was carried out on tissue sections stained with hematoxylin and eosin and subtyping was established according to the 2008 WHO classification (Swerdlow et al. 2008). The inclusion criteria for the retrospective study were a reference diagnosis of DLBCL and the availability of formalin-fixed and paraffin-embedded samples for immunohistochemical (IHC) evaluation. Clinical data on survival were not available from 12 other patients and were not included in the study. The analysis of archival lymph nodes samples was approved by the local Ethics Committee.

IHC Staining and Microscopic Evaluation

The IHC panel consisted of monoclonal mouse anti-human antibodies (Dako, Denmark) raised against CD20 (clone L26), CD10 (clone 56C6), BCL6 protein (clone PG-B6p),

IRF4/MUM1 (clone MUM1p), Ki-67 (clone MIB-1), as well as HLA-G (clone 4H84) from Exbio Antibodies (Czech Republic), and MHC class II including HLA-DP, HLA-DQ and HLA-DR (clone CR3/43) with the assumption that the latter would be more representative of overall tumor biology than HLA-DR alone. HLA-G (clone 4H84) is the anti-pan-HLA-G which detects heavy chains of all HLA-G isoforms of human origin, and moreover, is the only anti-HLA-G monoclonal antibody that works by IHC on paraffin-embedded tissue sections (LeMaoult et al. 2003). A polyclonal rabbit anti-human antibody against CD3 was used (Dako, Denmark). All immunostainings were independently evaluated by two experienced hematopathologists who were blinded to patient outcome. Staining for CD20 and CD3 was scored as positive or negative, and staining for BCL6, IRF4/MUM1 was considered positive if 30 % or more of the tumor cells were stained with an antibody according to a uniform cut-off based on prior analysis and publications (Hans et al. 2004). All cases were assigned to the GCB (Germinal Center B-cell like) or non-GCB group as proposed by Hans et al. (2004). For Ki-67, the number of positive tumor cells was evaluated by visual estimation and recorded in 10 % increments.

In summary, paraffin-embedded sections were deparaffinised with xylene (2 × 5 min) and dehydrated with ethanol (×1 min 100 % ethanol, 1 × 1 min 96 % ethanol). Subsequently, the slides were rinsed with peroxide. For antigen retrieval, the HIER (heat-induced epitope retrieval) method was used according to the manufacturer's protocol (PT Module Deparaffinization and Heat-Induced Epitope Retrieval Solutions, Thermo Scientific, USA). Primary mouse monoclonal antibody anti-HLA-G (dilution 1:100) was incubated for 3 h (Paul et al. 2000) and, simultaneously, primary mouse monoclonal antibody anti-HLA class II (dilution 1:100) was incubated for 30 min at room temperature. The sections were then rinsed with buffer (Tris buffered & saline Tween 20; Dako, Denmark). For detection and visualization, an EnVision System HRP (Dako, Denmark) was used following the manufacturer recommendations. All sections were counterstained with Harris hematoxylin (Instant Hematoxylin, Bio-Optica, Italy). Microscopic evaluation (Olympus, BX63) of HLA-G and HLA class II immunostaining was performed at 200× and 400× magnification. HLA-G immunoreactivity was determined in a binary manner based on the percentage of HLA-G positive tumor cells. Cases were considered positive when more than 25 % of lymphoma cells expressed intermediate/strong membranous staining of HLA-G as described previously (He et al. 2010; Jeong et al. 2014). The cut-off value for determining the positivity of HLA class II expression was 10 % of the staining area, irrespective of staining intensity based on previous publications (Schmelz et al. 2012; Wilkinson et al. 2012). The intensity of staining was not used to determine positivity because the variability in tissue fixation and processing appeared to affect the intensity of staining. Results were compared for discrepant cases (<10 % of observations), reviewed, and a consensus interpretation reached. The histopathological images with positive and negative HLA-G or HLA class II staining along with the corresponding CD20 staining and isotype specific control staining are, respectively, presented in Figs. 1, 2. The immunohistochemistry was validated using a positive and negative tissue control in all series of immunostained slides. For validation of HLA-G staining, positive controls (with tissues containing the target antigen at known and stable expression level) on human trophoblast and reactive lymph nodes tissue were performed. The controls for MHC class II staining were performed on thymus and spleen, for other antibodies tonsil tissue and reactive lymph nodes were used. For negative control staining, placenta and examined neoplastic lymph nodes were evaluated using mouse isotype antibody Ready-to-Use FLEX Negative Control Mouse (Cocktail of mouse IgG1, IgG2a, IgG2b, IgG3 and IgM, IR750; Dako, Denmark) (Fig. 2m–o). Additionally, positive internal controls containing the cells with target antigen within normal tissue elements were also assessed during the analysis. For example, typical staining for CD20, BCL2, BCL6, CD10, MUM-1 and Ki-67 was observed in DLBCL cases in residual follicles with germinal centers, and macrophages were stained with HLA class II. In some cases, very weak staining of HLA-G in small cells in the background of DLBCL infiltration was observed, and among them were T lymphocytes (Fig. 1g), which was compared with the expression of CD3 in adequate tissue sections. However, IHC double staining for indicating HLA-G/CD3 positive cells was not performed, since both the size and morphology of DLBCL cells allow identification of intermediate/strong membranous staining of HLA-G on lymphoma cells (Fig. 1g). This observation is consistent with the previous study indicating occasional very weak staining of HLA-G in nearby histiocytes and lymphocytes (Diepstra et al. 2008).

Treatment

Of the 148 patients, first-line treatment was given to 143 subjects. The ECOG status of the five remaining patients was so poor that they were only allocated steroid therapy. Of the remaining 143 patients, 109 (76 %) received six or eight cycles of either immunochemotherapy according to the R–CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone) or R–COP (rituximab, cyclophosphamide, vincristine, and prednisolone) regimen. The latter regimen was given to four (3 %) patients due to

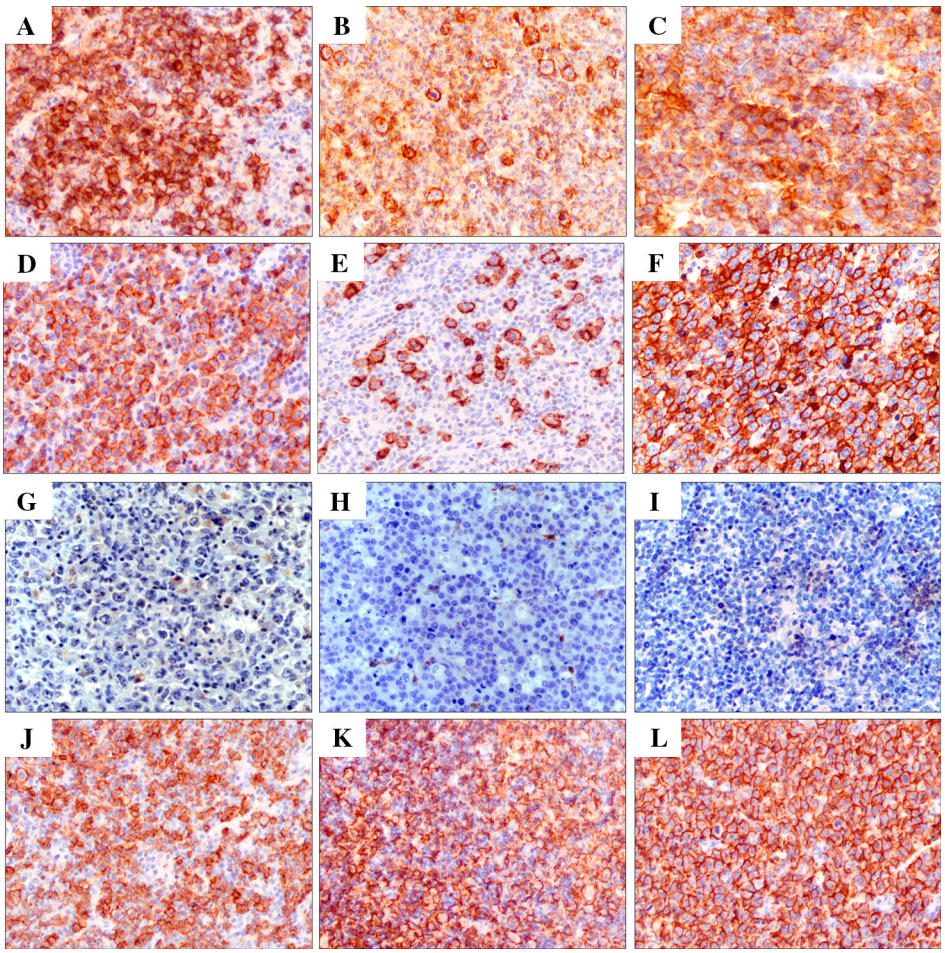

Fig. 1 **a–c** Representative results of three DLBCL cases with the positive HLA-G expression in over 25 % of neoplastic cells. **d–f** Anti-CD20 staining corresponding with the cases of the positive HLA-G expression. **b**, **e** Demonstrate T-cell/histiocyte rich DLBCL (TCRBCL) with scattered large neoplastic cells. **g–i** Representative results of three cases with DLBCL with the negative HLA-G expression in neoplastic cells. Please note that some small cells in the background are HLA-G positive; **j–l** Anti-CD20 staining corresponding with the cases of the negative HLA-G expression. Haematoxylin counterstaining, original magnification: ×200

their comorbid diseases and poor performance status. The remaining 34 (24 %) patients were treated with CHOP-like regimens and their clinical characteristics did not differ from those receiving immunochemotherapy.

After completing the treatment, the patients were followed every 3 months during the first year, then every 6 months for two additional years, and then yearly afterwards. Follow-up information included the response to initial therapy based on criteria of the International Workshop to Standardize Response Criteria for Non-Hodgkin Lymphomas (Cheson et al. 1999).

Statistical Analysis

Associations between HLA-G and HLA class II expression and clinical or biological variables were assessed with the χ^2 test, and, if required the Fisher's exact test was used. Receiver operating characteristic curves were used to provide the specific cut-off points of Ki-67 expression. Logistic regression was used to estimate the effect of the HLA-G and HLA class II expression on the complete remission (CR) rate. Progression-free survival (PFS) was determined from the onset of treatment until relapse, disease progression, or the last follow-up evaluation. Overall survival (OS) was determined from the date of diagnosis until last follow-up or death from any cause. Survival (PFS and OS) was estimated by the Kaplan–Meier method and compared using the log-rank test. 95 % confidence intervals (95 % CI) were calculated. The Cox proportional hazard model adjusted for the IPI factors was used to estimate the potential effect of HLA-G and HLA class II expression on PFS and OS. Statistical tests with $p < 0.05$ were considered significant. All reported p values are two-sided. Statistical analysis was performed using the SPSS 15.0 package (SPSS Inc., Chicago, IL, USA).

Results

HLA-G and HLA Class II Expression in DLBCL and Their Association with Prognostic Variables

The positive expression of HLA-G was observed in 36 patients (24 %), whereas HLA class II was found in 110 patients (74 %) with DLBCL at the time of diagnosis. The staining results for the analyzed IHC markers are presented

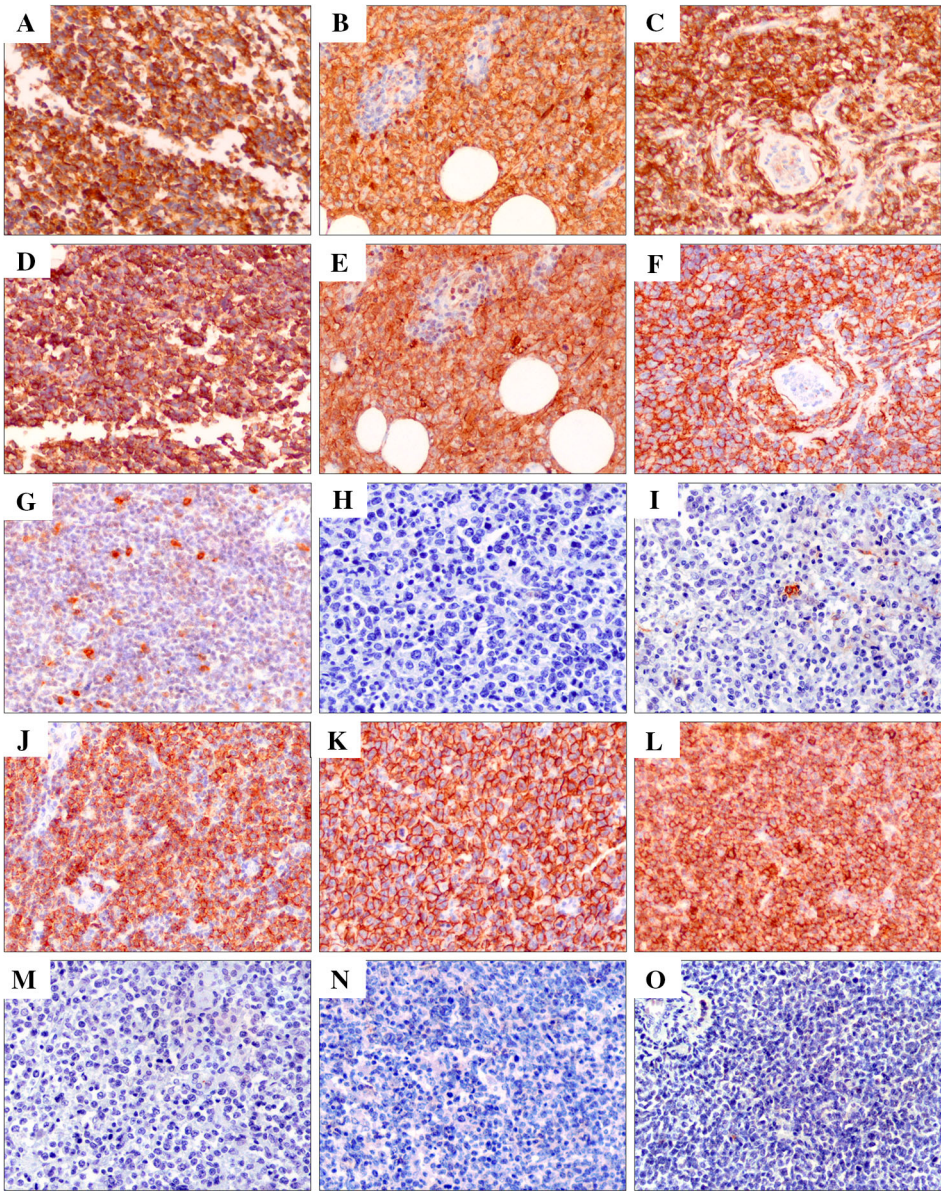

Fig. 2 **a–c** Representative results of three cases with DLBCL with the positive HLA class II expression in over 10 % of neoplastic cells. **d–f** Anti-CD20 staining corresponding with the cases of the positive HLA class II expression. **g–i** Representative results of three cases with DLBCL with the negative HLA class II expression in neoplastic cells. A few small cells (mainly macrophages) in the background are HLA class II positive. **j–l** Anti-CD20 staining corresponding with the cases of the negative HLA class II expression. Isotype specific control staining for HLA-G (**m**), HLA class II (**n**) and CD20 (**o**). Haematoxylin counterstaining, original magnification: ×200

in Table 1. For the study group, 58 patients (39 %) were assigned to the GCB and 90 subjects (61 %) to the non-GCB group.

The association between the expression of HLA-G and HLA class II and prognostic factors incorporated in the IPI, as well as other disease features known to correlate with the clinical prognosis of DLBCL, were analysed (Table 2). The positive expression of HLA-G was observed more frequently in the patients with a serum albumin level greater than 35.0 g/L, compared with the negative HLA-G expression in the patients with low albumin levels (79 vs. 55 %; $p = 0.02$). A trend towards a higher frequency of positive HLA-G expression in young patients was noted compared to patients aged over 60 with negative HLA-G expression (61 vs. 56 %; $p = 0.08$). The positive expression of HLA class II was found more frequently in females, whereas its negative expression was mostly seen in male patients (62 vs. 58 %; $p = 0.04$).

No association was found between the expression of HLA-G and HLA class II in the study group (Table 3). HLA-G expression was also similar in the two different subtypes of DLBCL. However, a positive association between HLA class II and IRF4/MUM1 expression was shown ($p = 0.04$; Table 3).

HLA-G and HLA Class II Expression in Relation to Response to First-Line Treatment

After the induction therapy, 88 patients (62 %) achieved a CR, whereas 55 (39 %) did not. In the group treated with

Table 1 Summary of immunohistochemical markers in 148 patients with DLBCL at the time of diagnosis

Characteristics	*N* (%)
HLA-G	
Positive	36 (24)
Negative	112 (76)
HLA class II	
Positive	110 (74)
Negative	38 (26)
BCL6	
Positive	90 (62)
Negative	55 (38)
Unknown	3
CD10	
Positive	30 (22)
Negative	109 (78)
Unknown	9
IRF4/MUM1	
Positive	75 (57)
Negative	56 (43)
Unknown	17
BCL2	
Positive	100 (72)
Negative	39 (28)
Unknown	9
Ki-67	
<80 %	65 (49)
≥80 %	68 (51)
Unknown	15
DLBCL subtypes	
GCB	58 (39)
Non-GCB	90 (61)

N number of patients

immunochemotherapy, 69 patients (63 %) had a remission and in the group receiving chemotherapy 19 subjects (56 %) did so. Since no significant difference was observed in the response rate between the aforementioned groups ($p = 0.7$, test χ^2), the potential impact of the analyzed variables on the response to treatment was assessed in the whole group of patients. After completion of the first-line treatment, 49 % of patients (43/88) experienced disease relapse or progression. The median time to relapse or progression was 35 months (95 % CI 15–55 months).

In univariate logistic regression analysis, the negative HLA-G expression was associated with a lower probability of achieving a CR ($p = 0.04$) (Table 4A). Other potential prognostic factors that showed an adverse impact on CR achievement were the intermediate high/high IPI risk group ($p = 0.02$; Table 4A), Ann Arbor advanced disease stage [odds ratio (OR) 0.3, 95 % CI 0.1–0.9; $p = 0.03$], elevated LDH levels (OR 0.3, 95 % CI 0.1–0.7; $p = 0.01$), elevated β2-microglobulin levels (OR 0.3, 95 % CI 0.1–0.96; $p = 0.04$), the presence of B symptoms (OR 0.3, 95 % CI 0.2–0.8; $p = 0.008$), and low serum albumin levels (OR 0.2, 95 % CI 0.08–0.7; $p = 0.007$).

Multivariate logistic regression analysis including all the above listed parameters revealed that only the low serum albumin level negatively influenced the CR achievement (OR 0.2, 95 % CI 0.04–0.7; $p = 0.016$). An additional multivariate analysis adjusted for the IPI risk groups and the HLA-G expression showed that only the IPI retained its independent impact on achieving a CR. It is also worth mentioning that the statistical trend towards an independent influence of HLA-G expression on CR achievement was observed (Table 4B).

There was no impact of HLA-G or HLA class II expression on the probability of PFS (data not shown).

HLA-G and HLA Class II Expression in Relation to Outcome

Of all the 148 patients, 66 (45 %) died. The median time of OS was 46.8 months (95 % CI 28–65 months).

The patients with the positive HLA-G expression presented a trend towards a higher 3-year OS rate compared to those with negative expression of HLA-G (69.2 %, 95 % CI 50–83, vs. 48.4 %, 95 % CI 38–59; $p = 0.08$, respectively) (Table 5A; Fig. 3a).

The estimated 3-year OS rate of patients with positive HLA class II expression was 59.4 % (95 % CI 49–69) in comparison to 37.4 % (95 % CI 22–56; $p = 0.04$) in subjects with the loss of HLA class II expression (Table 5A; Fig. 3b). Other prognostic factors observed to influence a shorter OS were the intermediate high/high IPI risk group ($p = 0.001$; Table 5A), ECOG performance status ≥2 ($p < 0.0001$), the number of extranodal sites ≥2 ($p = 0.03$), elevated LDH levels ($p < 0.0001$), elevated β2-microglobulin levels ($p = 0.001$), the presence of B symptoms ($p = 0.01$), anemia ($p = 0.02$) and low serum albumin levels ($p < 0.0001$).

A multivariate Cox analysis including all the above listed parameters revealed that only the IPI risk groups retained their independent prognostic impact on OS (OR 12.9, 95 % CI 1.7–98.4; $p = 0.01$). An additional multivariate Cox analysis adjusted for the IPI risk groups showed that both the intermediate high/high IPI risk group ($p = 0.001$) and the loss of HLA class II expression ($p = 0.05$) independently increased the risk of death in the study group (Table 5B).

We also investigated whether the impact of HLA class II expression on OS may be related to the subtypes of DLBCL. In the subgroup of 58 patients (39 %) with GCB-type pattern, the patients with the loss of HLA class II expression

Table 2 The association of HLA-G and HLA class II expression with other prognostic parameters in 148 patients with DLBCL at the time of diagnosis

Characteristic	HLA-G positive *N* (%)	HLA-G negative *N* (%)	*p*	HLA class II positive *N* (%)	HLA class II negative *N* (%)	*p*
Sex						
Female	20 (56)	64 (57)	1.0	68 (62)	16 (42)	0.04
Male	16 (44)	48 (43)		42 (38)	22 (58)	
Age						
60 years or younger	22 (61)	49 (44)	0.08	53 (48)	18 (47)	1.0
Older than 60 years	14 (39)	63 (56)		57 (52)	20 (53)	
Performance status (ECOG score)						
Less than 2	21 (66)	56 (54)	0.3	56 (58)	21 (55)	0.7
2 or more	11 (34)	47 (46)		41 (42)	17 (45)	
Unknown	13			13		
Ann arbor stage						
I, II	11 (32)	24 (24)	0.4	28 (28)	7 (21)	0.5
III, IV	24 (68)	76 (76)		73 (72)	27 (79)	
Unknown	13			13		
No. of extranodal sites						
Less than 2	29 (84)	82 (81)	0.8	84 (84)	27 (77)	0.3
2 or more	5 (16)	19 (19)		16 (16)	8 (23)	
Unknown	13			13		
Serum LDH level						
1× or less than normal	10 (33)	28 (27)	0.6	31 (31)	8 (22)	0.4
Greater than 1× normal	20 (67)	77 (73)		68 (69)	28 (78)	
Unknown	13			13		
Serum β2-microglobulin level						
1× or less than normal	11 (33)	28 (27)	0.8	26 (25)	12 (36)	0.4
Greater than 1× normal	22 (67)	77 (73)		79 (75)	21 (64)	
Unknown	10			10		
Bulky tumor (10 cm)						
Absent	27 (77)	69 (70)	0.6	73 (68)	28 (80)	0.2
Present	8 (23)	29 (30)		34 (32)	7 (20)	
Unknown	6			6		
B symptoms						
Absent	21 (58)	53 (55)	1.0	57 (58)	18 (51)	0.5
Present	15 (42)	44 (45)		41 (42)	17 (49)	
Unknown	15			15		
Serum albumin level						
Greater than 35.0 g/L	26 (79)	47 (45)	0.02	52 (51)	21 (58)	0.8
35.0 g/L or less	7 (21)	57 (55)		49 (49)	15 (42)	
Unknown	11			11		
Hemoglobin level						
Greater than 12 g/dL	17 (52)	53 (51)	1.0	51 (52)	18 (47)	0.7
12 g/dL or less	16 (48)	51 (49)		48 (48)	20 (53)	
Unknown	11			11		
IPI risk groups						
Low/intermediate low	18 (54.5)	43 (42)	0.2	46 (46.5)	15 (42)	0.7
Intermediate high/high	15 (45.5)	59 (58)		53 (53.5)	21 (58)	
Unknown	13			13		

χ^2 test: *p* values are two-sided, *N* number of patients

Table 3 The associations of HLA-G and HLA class II expression with other antigens estimated by IHC staining in 148 patients with DLBCL at the time of diagnosis

Characteristics	HLA-G positive *N* (%)	HLA-G negative *N* (%)	*p*	HLA class II positive *N* (%)	HLA class II negative *N* (%)	*p*
HLA class II						
Positive	28 (78)	82 (73)	0.7	–	–	–
Negative	8 (22)	30 (27)				
BCL6						
Positive	21 (60)	69 (63)	0.8	71 (66)	19 (51)	0.2
Negative	14 (42)	41 (37)		37 (34)	18 (49)	
Unknown	3			3		
CD10						
Positive	6 (17)	24 (23)	0.6	21 (19)	9 (30)	0.2
Negative	29 (83)	80 (77)		88 (81)	21 (70)	
Unknown	9			9		
IRF4/MUM1						
Positive	23 (68)	52 (54)	0.2	62 (63)	13 (41)	0.04
Negative	11 (32)	45 (46)		37 (37)	19 (59)	
Unknown	17			17		
BCL2						
Positive	26 (74)	74 (71)	0.8	76 (74.5)	24 (65)	0.3
Negative	9 (26)	30 (29)		26 (25.5)	13 (35)	
Unknown	9			9		
Ki-67						
<80 %	15 (44)	50 (50.5)	0.5	51 (50.5)	14 (44)	0.5
≥80 %	19 (56)	49 (49.5)		50 (49.5)	18 (56)	
Unknown	15					
DLBCL subtypes						
GCB	11 (31)	47 (42)	0.2	38 (34.5)	20 (53)	0.06
Non-GCB	25 (69)	65 (58)		72 (65.5)	18 (47)	

χ^2 test: *p* values are two-sided, *N* number of patients

presented a significantly lower 3-year OS rate than those with its positive expression [26 % (95 % CI 10–53) vs. 68.2 % (95 % CI 51–81); $p = 0.02$) (Fig. 4a). In contrast, in the subgroup of 90 non-GCB patients (61 %), HLA class II expression did not influence OS (Fig. 4b).

To further explore the unexpectedly favorable effect of positive HLA-G expression on the clinical course of DLBCL, an additional analysis was performed that considered the type of treatment (chemotherapy with or without rituximab). In the group of patients treated with immunochemotherapy, a more pronounced effect of the positive HLA-G expression on OS was revealed. The estimated 3-year OS rate of patients with the positive HLA-G expression was 73.3 % (95 % CI 49–88) compared to 47.5 % (95 % CI 35–60; $p = 0.03$) in subjects with the negative HLA-G expression (Fig. 5a). In contrast, in the group treated with CHOP-like regimens, no significant impact of HLA-G expression on OS was observed (Fig. 5b); however, an opposite tendency for better survival in patients with the negative HLA-G expression over the first three years should be noted; a 3-year OS rate for HLA-G positivity was 20 % (95 % CI 4–62) vs. 55.3 % (95 % CI 37–72; $p = 0.08$) for the absence of HLA-G. Additionally, the prognostic value of HLA class II expression was also shown to depend on the use of rituximab as a part of first-line treatment. In the patients receiving immunochemotherapy, those that had positive HLA class II expression demonstrated a 3-year OS rate of 65.3 % (95 % CI 52–76) compared to 29.6 % (95 % CI 13–53; $p = 0.04$) in subjects with the loss of HLA class II expression (Fig. 6a). However, HLA class II expression did not have a prognostic impact on OS in the patients treated with chemotherapy alone; the 3-year OS rate was 49.5 % (95 % CI 32–67) in the subjects with positive expression compared with 50 % (95 % CI 15–85; $p = 0.8$) in those with the loss of HLA class II expression (Fig. 6b).

Table 4 Probability of achieving complete remission using univariate (A) and multivariate (B) logistic regression analysis

A			
Characteristics	*N*	OR (95 % CI)	*p*
HLA-G			
Positive	33	1.0	0.04
Negative	110	0.4 (0.1–1.0)	
HLA class II			
Positive	109	1.0	0.8
Negative	34	0.9 (0.4–2.1)	
DLBCL subtypes			
GCB	58	1.0	0.9
Non-GCB	85	1.0 (0.5–2.0)	
IPI risk groups			
Low/intermediate low	61	1.0	0.02
Intermediate high/high	71	0.4 (0.2–0.9)	
B			
Factors	*N*	OR (95 % CI)	*p*
HLA-G			
Positive	30	1.0	0.058
Negative	102	0.4 (0.1–1.0)	
IPI risk groups			
Low/intermediate low	61	1.0	0.04
Intermediate high/high	71	0.4 (0.2–0.96)	

N number of patients, *OR* odds ratio, *95 % CI* 95 % confidence interval

Table 5 Probabilities of 3-year overall survival of 148 patients with DLBCL in univariate (A) and multivariate (B) analysis

A			
Characteristics	*N*	OR (95 % CI)	*p*
HLA-G			
Positive	36	69.2 (50–83)	0.08
Negative	112	48.4 (38–59)	
HLA class II			
Positive	110	59.4 (49–69)	0.04
Negative	38	37.4 (22–56)	
DLBCL subtypes			
GCB	58	54 (39–68)	0.7
Non-GCB	90	53 (41–65)	
IPI risk groups			
Low/intermediate low	61	67.5 (53–79)	0.001
Intermediate high/high	74	41.7 (29–55)	
B			
Factors	*N*	RR (95 % CI for RR)	*p*
HLA class II			
Positive	99	1.0	0.05
Negative	36	1.7 (1.0–2.8)	
IPI risk groups			
Low/intermediate low	61	1.0	0.001
Intermediate high/high	74	2.4 (1.4–4.2)	

N number of patients, *OS* overall survival, *RR* relative risk, *95 % CI* 95 % confidence interval, *p* groups were compared using the log-rank test

Discussion

To the best of our knowledge, this report is the first to describe the expression of HLA-G protein in DLBCL tissues at the time of diagnosis in relation to the clinical course of the disease. Moreover, we assessed the impact of MHC class II expression on the outcome of the patients according to the subtypes of DLBCL and the type of treatment.

So far, only a few studies have investigated the HLA-G protein expression on lymphoma cells (Diepstra et al. 2008; Urosevic et al. 2002, 2004). It has been reported that *HLA-G* is transcribed in B- and T-cell populations, and has also been detected in cases of B-NHL suggesting that HLA-G is a marker of mature lymphoid cells and may play a role in lymphomagenesis (Amiot et al. 1996). Urosevic et al. (2002) detected HLA-G protein expression by IHC in 51 % of patients (23/45) with primary cutaneous lymphomas, including 7/10 cases of cutaneous B-cell lymphoma (CBCL) and 16/35 T-cell lymphomas (CTCL). Interestingly, it was generally the indolent lymphomas that presented HLA-G positivity, whereas in CTCL the positive expression of HLA-G correlated with high-grade histology and advanced stage of the disease. It is worth noting that among ten cases of CBCL, five were diagnosed as DLBCL and only in one patient (20 %) was single-cell immunoreactivity with anti-HLA-G noted. Moreover, elevated interleukin (IL)-10 expression was detected in 73 % of the HLA-G positive cases suggesting that IL-10 was responsible for HLA-G up-regulation in CLL (Urosevic et al. 2002). In the next study by Urosevic et al. (2004), seven cases of CTCL were analyzed. HLA-G protein was expressed in two out of three lymphomas of the $CD56^+CD4^+$ type and by all lymphomas of the $CD8^+$ type. It was found that $CD56^+CD4^+$ lymphomas displayed stronger expression of HLA-G than the $CD8^+$ cases. Interestingly, both $CD56^+CD4^+$ and $CD8^+$ lymphomas expressed ILT2, the specific inhibitory receptor of HLA-G. HLA-G protein was also expressed by Hodgkin–Reed–Sternberg (HRS) cells in 54 % of patients (95/175) with classical Hodgkin lymphoma (Diepstra et al. 2008). In contrast, in the non-classical nodular lymphocyte-predominant subtype, neoplastic cells demonstrated HLA-G expression only in one

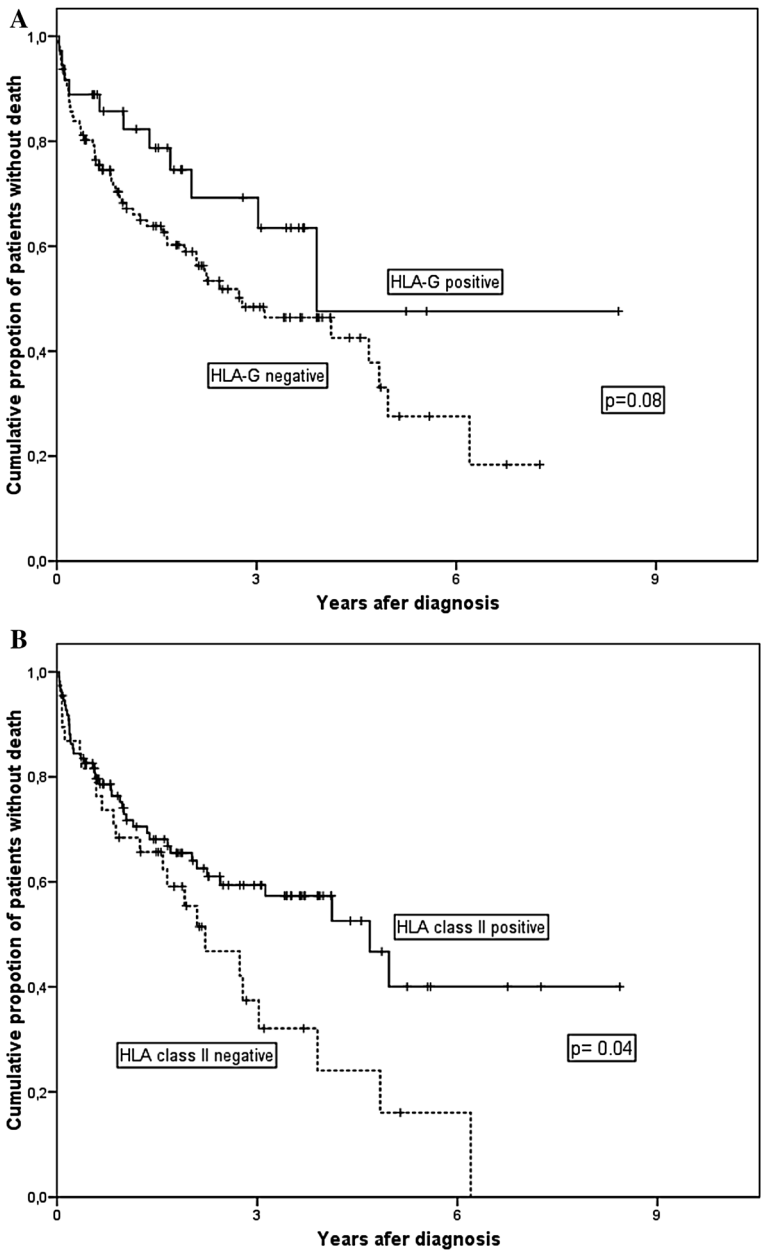

Fig. 3 Overall survival of 148 patients with DLBCL according to the initial expression of HLA-G (**a**) or HLA class II (**b**)

out of ten cases (Urosevic et al. 2002). Interestingly, Sebti et al. (2007) observed a significant increase in sHLA-G expression in the T-NHL in vitro model after cytokine stimulation; however, the different combinations of cytokines had no effect on its expression in B-NHL. Only after adding normal haematopoietic cells, such as monocytes, was the increased sHLA-G secretion seen. This may suggest that the lymph node microenvironment consisted of haematopoietic cells accompanied by a stromal compartment, has an impact on HLA-G expression in B-NHL. In our study, the positive expression of HLA-G on DLBCL tissue sections was detected in 24 % of patients. The cut-off value for positivity was assumed as being more than 25 % of lymphoma cells expressed in intermediate/strong membranous staining of HLA-G based on previous studies of breast cancer patients (He et al. 2010; Jeong et al. 2014). In comparison, in the study by Urosevic et al. (2002), the positive expression of HLA-G was described as a strong positivity (numerous cells dispersed within the malignant infiltrate) or as a single-cell positivity (scant, scattered cells throughout the infiltrate) where the strong HLA-G expression was detected in half of the positive cases in both CBCL and CTCL lymphomas. On the other hand, HLA-G staining, diffusely cytoplasmic, was deemed positive when at least 50 % of neoplastic cells showed stronger staining than occasional positive bystander cells (Diepstra et al. 2008).

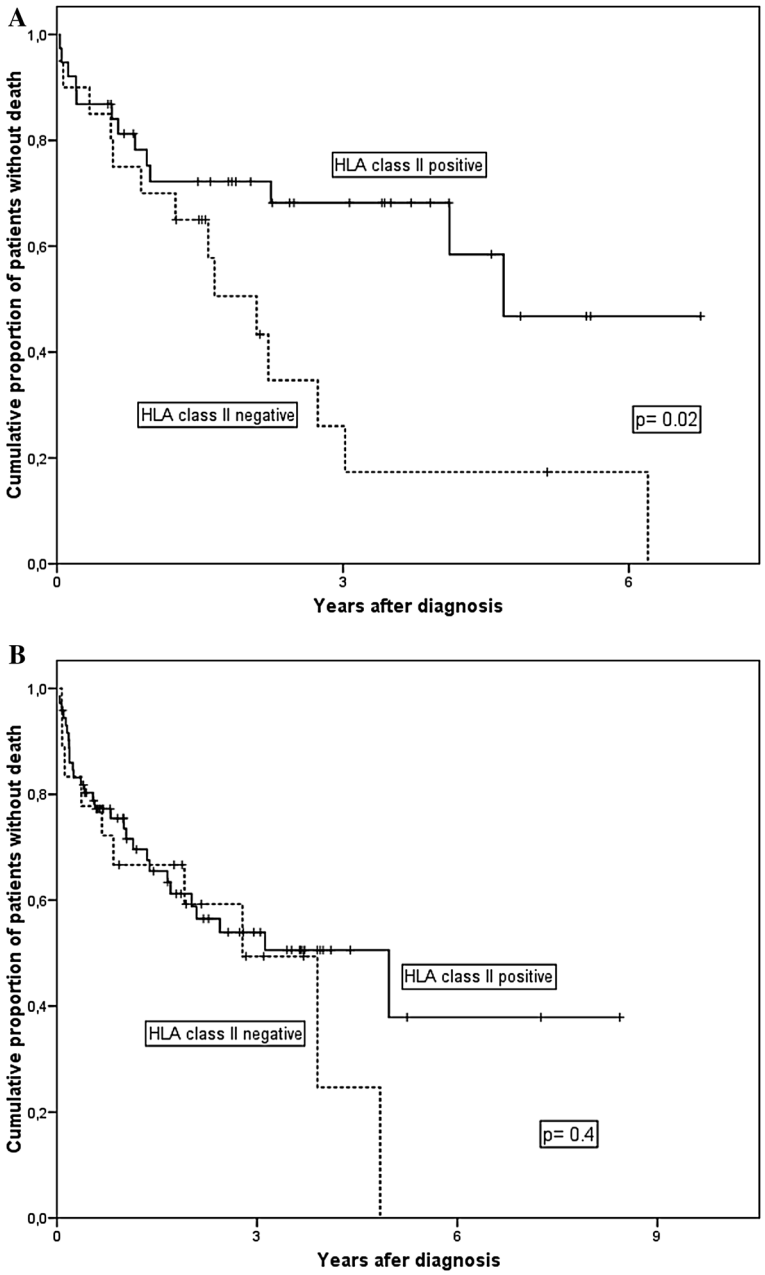

Fig. 4 Overall survival of 148 DLBCL patients with the GCB (**a**) or non-GCB (**b**) subtype according to the initial expression of HLA class II

In our study, it was unexpectedly observed that the patients with positive HLA-G expression had a higher probability of achieving a CR and exhibited a tendency to longer OS than subjects with negative HLA-G expression. Additionally, the positive expression of HLA-G protein was associated with normal serum albumin levels and more often, but without statistical significance, was seen in patients who were 60 years or younger. The expression and clinical significance of HLA-G however remains controversial both in hematologic malignancies and in solid tumors (Loustau et al. 2013; Yan 2010) In some studies, the high expression of HLA-G on circulating CLL cells was associated with adverse outcome (shorter PFS or treatment-free survival) in patients with CLL compared to those with low HLA-G expression (Nuckel et al. 2005; Rebmann et al. 2007; Rizzo et al. 2014). On the other hand, it was reported that neither mRNA expression of HLA-G nor sHLA-G had prognostic value in patients with CLL and NHL (Giannopoulos et al. 2008; Sebti et al. 2007; Park et al. 2008). However, in the above-mentioned studies, different methods were used to evaluate HLA-G expression in lymphoma patients therefore their possible impact on different results as well as their incomparability should be also taken into account. In the context of solid tumors,

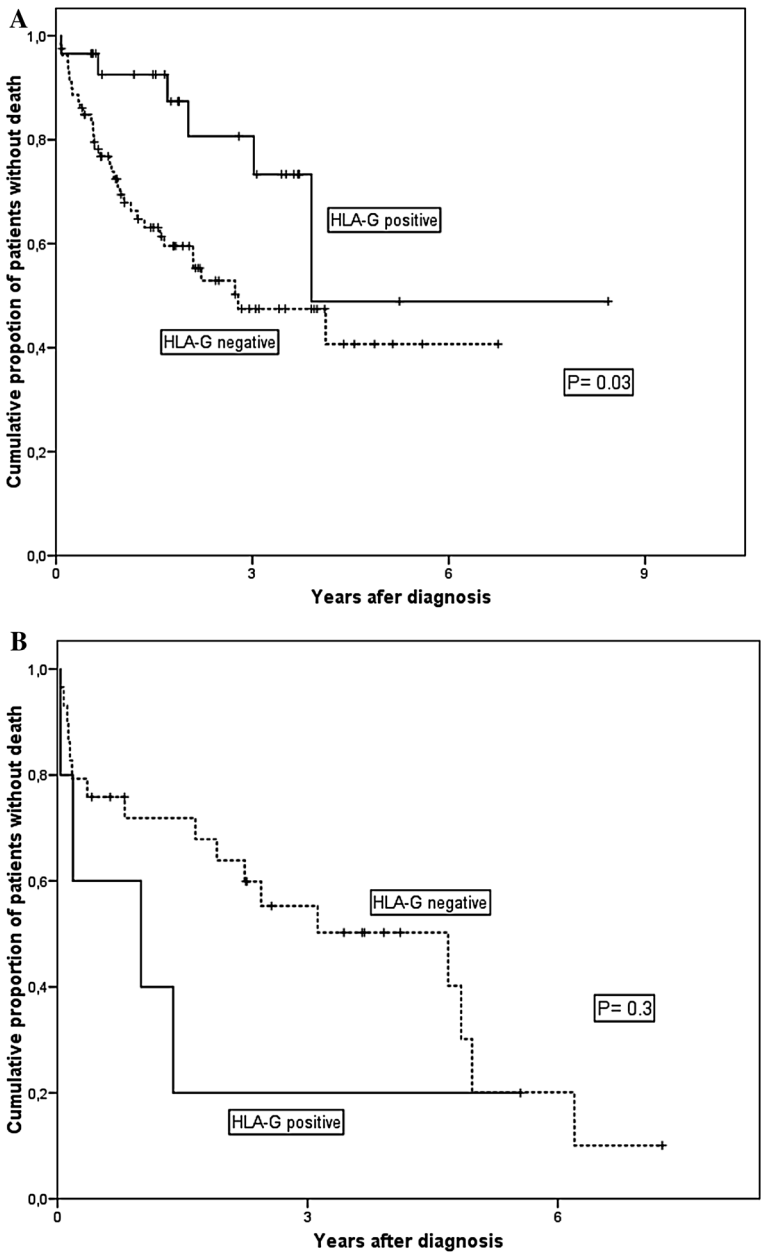

Fig. 5 Overall survival of 148 DLBCL patients treated with R–C(H)OP (**a**) or CHOP-like (**b**) according to the initial expression of HLA-G. *R–C(H)OP* rituximab, cyclophosphamide, doxorubicin±, vincristine, prednisolone, *CHOP* cyclophosphamide, doxorubicin, vincristine, prednisolone

aberrant expression of HLA-G was mainly detected in aggressive cancers and was associated with an unfavorable outcome of the disease. It is hypothesized that HLA-G, due to its immune inhibitory activity, induces escape from host immunosurveillance and helps malignant cells survive (Carosella et al. 2008; Larsen and Hviid 2009; Loustau et al. 2013). However, in the recent study by Rutten et al. (2014), the up-regulation of HLA-G expression measured by IHC in the patients with high-grade epithelial ovarian carcinomas correlated with longer PFS and response to chemotherapy, as well as being an independent parameter for improved survival. In this elegant study, it was also revealed that elevated gene expression of HLA-G was associated with good prognosis; however, no correlation was observed between protein expression of HLA-G in tumor tissue and HLA-G gene expression (Rutten et al. 2014). In our previous study, it was shown that the patients with DLBCL carrying the high risk *HLA-G* genotypes presented a shorter OS than those of the low-risk group (Bielska et al. 2015). We performed an additional analysis to further examine any possible associations between the expression of HLA-G protein in DLBCL tissues and functionally relevant *HLA-G* polymorphisms, namely *HLA-G*-725C/G/T and *HLA-G* 14-bp; however, no correlation was observed between the HLA-G expression and *HLA-G* genotype-based risk groups.

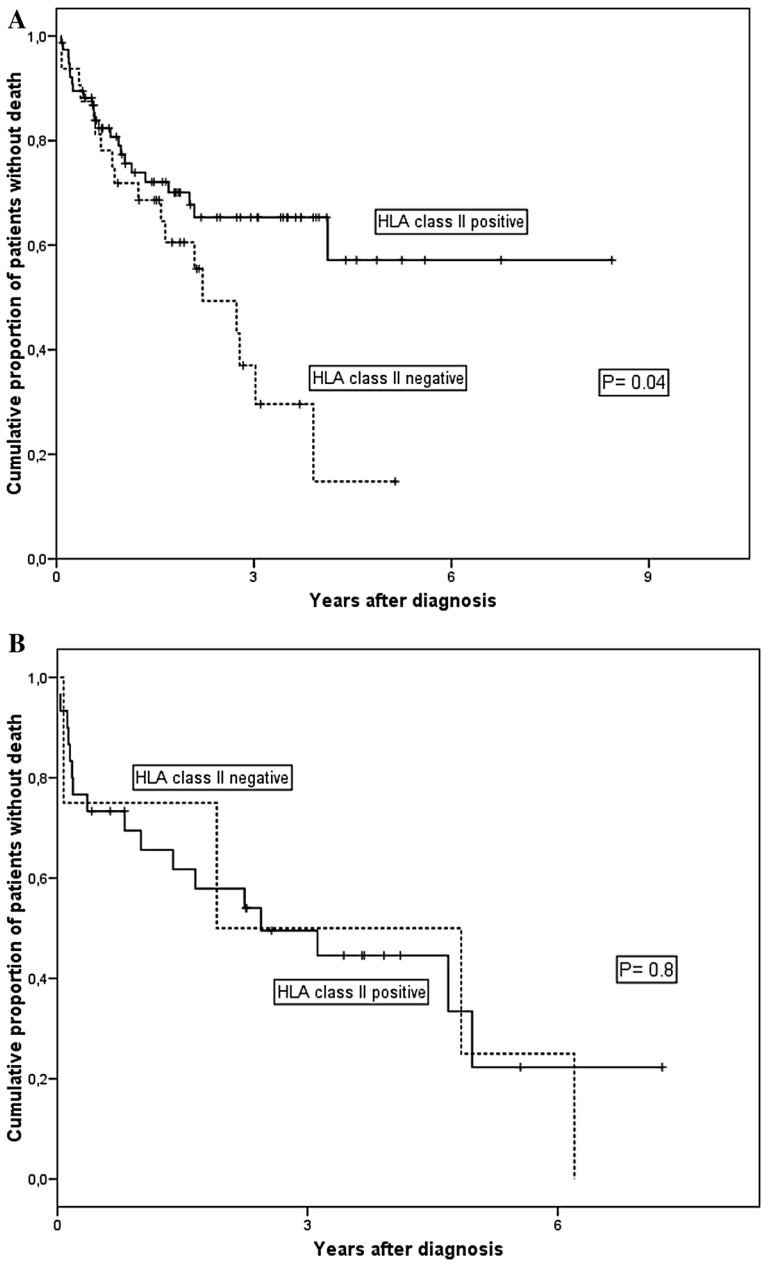

Fig. 6 Overall survival of 148 DLBCL patients treated with R-C(H)OP (**a**) or CHOP-like (**b**) according to the initial expression of HLA class II. *R-C(H)OP* rituximab, cyclophosphamide, doxorubicin±, vincristine, prednisolone. *CHOP* cyclophosphamide, doxorubicin, vincristine, prednisolone

Since our aforementioned findings are in contrast to some studies reporting the adverse clinical impact of HLA-G positivity in hematological malignancies, it led us to a question whether the anti-CD20 antibody, rituximab, which is known to modulate the prognostic value of some biological markers may have any effect on the clinical significance of HLA-G expression. We demonstrated that in the patients treated with immunochemotherapy, the positive expression of HLA-G still correlated with longer OS. In contrast, in the subjects receiving chemotherapy alone, no significant effect on OS was achieved; however, a tendency for better survival in patients with the absence of HLA-G during the first three years compared to those with the positive HLA-G expression should be noted. This observation remained opposite to that shown in the patients receiving immunochemotherapy. The lack of statistical association could, however, be explained by the low number of patients ($n = 34$) treated with chemotherapy, especially in the subgroup with positive expression of HLA-G ($n = 5$). It was also previously reported by Inagaki et al. (2009) that lymphoma cell susceptibility to rituximab-induced antibody-dependent cellular cytotoxicity (ADCC) was determined by three major tumor-associated factors, namely the amount of CD20 antigen, the amount of

ligands for inhibitory killer Ig-like receptors, such as classical MHC class I or HLA-G, and the amounts of some of the NKG2D ligands; especially UL16-binding protein (ULBP) 1-3. In this elegant study, an inverse correlation between the amount of MHC class I expression and the degree of rituximab-induced ADCC were shown; however, in the context of HLA-G expression in NHL cell lines, no significant correlations with rituximab-induced ADCC were observed. It was also reported that positive triggering of NK cells largely depended on the activating receptor NKG2D ligands such as ULBP as well as MICA and MICB non-classical MHC molecules which may overcome an inhibitory signal generated by KIRs stimulation (Inagaki et al. 2009). However, the precise mechanisms which render the lymphoma cells susceptible to rituximab-induced ADCC and the role of HLA-G and other non-classical HLA proteins in this process deserve further study. A possible explanation as to why positive HLA-G expression on DLBCL cells have favorable impact on OS could be provided by a study by Naji et al. (2012) showing that proliferation of lymphoma cells expressing ILT-2 receptor is inhibited by HLA-G due to a cell-cycle blockade. This observation remains opposite to that noted in some studies on solid tumors where HLA-G favors tumor escape because solid tumors cells do not express receptor to HLA-G (Carosella et al. 2008; Naji et al. 2012). However, in the study on melanoma cell lines, the expression of HLA-G rendered tumor cells more susceptible to NK cell-mediated lysis due to a switch in alternative splicing replacing the loss of cell surface HLA-G1 by intracellular HLA-G2 expression (Rouas-Freiss et al. 2005).

This would be of interest to investigate the HLA-G expression in relation to the expression of HLA class Ia antigens in DLBCL patients. It was previously reported that the loss of HLA class I molecules on lymphoma cells determined by flow cytometry correlated with more advanced disease and poor outcome in seven patients with NHL. Simultaneously, the absence of HLA-G protein expression was observed on the cell surface of all the patients; however, in some cases, the mRNA expression of transcripts encoding soluble forms of HLA-G was present (Amiot et al. 1998). On the other hand, Diepstra et al. (2008) observed a strong association between HLA-G positivity and the absence of MHC class Ia expression on the cell surface of HRS cells as well as with an Epstein Barr virus negative status. It was also reported in several studies that the positive expression of non-classical HLA molecules in solid tumors with the absence of HLA class Ia expression correlated with worse prognosis. It could be explained by the mechanism of downregulation of classical HLA class I resulting in escape of tumors from cytotoxic T-cell recognition, and moreover, by enhancing this immunotolerance due to up-regulation of non-classical HLA-G expression and subsequent inhibition of NK cell-mediated lysis (Bukur et al. 2012; Rutten et al. 2014).

The additional aim of our study was to assess the expression of HLA class II protein by IHC in DLBCL patients in relation to HLA-G expression. The loss of HLA class II expression in 26 % of patients was observed. In comparison, Rimsza et al. (2007) found the loss of HLA-DR expression in 37 % of cases; however, any positive staining for HLA-DR, even in a minority of cytologically malignant appearing cells was considered as a positive result. In contrast, previously published studies demonstrated a loss of MHC class II molecules in approximately 10 % of cases (Miller et al. 1988; Stopeck et al. 2000).

In our study, no association was found between HLA-G and HLA class II expression in DLBCL; however, we confirmed the previously reported unfavorable influence of the loss of HLA class II expression on survival in patients with DLBCL. This observation is in agreement with other studies indicating that the loss of MHC class II gene expression or HLA-DR protein expression correlated with adverse outcome in patients with DLBCL, primary mediastinal large B-cell lymphoma and plasmablastic lymphoma (Rimsza et al. 2004, 2006, 2007; Roberts et al. 2006; Schmelz et al. 2012; Wilkinson et al. 2012). HLA class II antigens are involved in cellular immune responses by participating in antigen presentation and stimulation of T cells, and thus the loss of their expression may promote tumor escape from immunosurveillance. In patients with DLBCL treated in two prospective randomized trials, the loss of HLA-DR expression measured by IHC staining remained an independent risk factor of shorter OS compared to those with positive HLA-DR expression (Bernd et al. 2009). Furthermore, the loss of HLA class II expression was reported to be frequent in DLBCL arising in immune-privileged sites such as the central nervous system and testis due to both homozygous and hemizygous deletions or mutations in *HLA-DR* and *HLA-DQ* genes as well as low levels of HLA-DR mRNA expression (Booman et al. 2006; Jordanova et al. 2003). It is worth noting that the lower expression of MHC class II genes was also observed in the clinically unfavorable ABC-DLBCL compared with GCB-DLBCL subtype using Affymetrix GEP data (Rimsza et al. 2004; Roberts et al. 2006). It seems that the downregulation of MHC class II expression in DLBCL may occur by multiple distinct mechanisms; however, according to the study by Cycon et al. (2009) decreases in transcriptional class II transactivator expression appear to be the most prevalent. It should be emphasized that the patients in the aforementioned studies received chemotherapy without rituximab as a front-line treatment (Rimsza et al. 2004, 2006, 2007; Roberts et al. 2006). In our study, we observed that the relevance of HLA class II expression to clinical outcome still remains

significant in patients treated with immunochemotherapy with rituximab. However, in the present study, the influence of HLA class II expression on OS was not significant in the subjects receiving chemotherapy alone. This could be explained by the relatively low number of patients in this subgroup ($n = 34$), especially those with the loss of HLA class II expression ($n = 4$). Interestingly, we also found that the adverse impact of the loss of HLA class II expression was related to the DLBCL subtypes, and retained its significance only in the patients with the GCB-type pattern. Altogether, it seems that the loss of MHC class II expression remains an important, independent prognostic factor in DLBCL.

To better understand the effect of the HLA-G and HLA class II antigens on lymphoma progression, a possible role of trogocytosis should also be taken into account. Trogocytosis involves all types of immune cells, including B cells, $CD8^+$, $CD4^+$, γ-δ T cells, and NK cells, while interacting with professional APCs (Caumartin et al. 2007; LeMaoult et al. 2007). It was observed that after HLA-DR and CD80 acquisition, T cells stimulated resting T cells in an antigen-specific manner, behaving as APCs themselves, whereas acquisition of HLA-G1 rendered T cells immunosuppressive. In a cancer setting, trogocytosis might also modulate the activity of immune system depending on the immune cells being involved. It was shown by Brown et al. (2012) that CD86 and HLA-G antigens were commonly acquired by T cells from malignant plasma cells. The presence of either CD86 or HLA-G on malignant cells was associated with a poor prognosis of the patients with multiple myeloma. Moreover, it was revealed that HLA-G^+ T cells had a regulatory potency similar to natural Tregs, thus providing a novel mechanism for MM to avoid effective immune surveillance (Brown et al. 2012). A recent study by LeMaoult et al. (2015) revealed that hematological tumor lines possess a trogocytic capability that allows them to capture membranes that contain the immune inhibitory molecule HLA-G from allogeneic as well as from autologous sources.

In conclusion, for the first time, the expression of HLA-G protein in DLBCL and its association with the clinical course of the disease as demonstrated and the link between the losing HLA class II protein expression with poor survival in patients treated with immunochemotherapy was confirmed. Although the clinical significance of the loss of HLA class II protein expression seems to be well understood, the contribution of HLA-G to the prognosis of B-cell malignancies deserves further study, especially its immunoregulatory functions in relation to treatment with rituximab, which remains an open question.

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Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

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