Published in final edited form as:

Leuk Lymphoma. 2009 November ; 50(11): 1830–1836. doi:10.3109/10428190903242628.

Human germinal center-associated lymphoma protein expression is associated with improved failure-free survival in Brazilian patients with classical Hodgkin lymphoma

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Abstract

The human germinal center-associated lymphoma (*HGAL*) gene has prognostic value in diffuse large B-cell lymphoma, and expression of its cognate protein is germinal center-specific. A previous study had suggested that *HGAL* protein expression might also be related to the outcome in patients with Hodgkin lymphoma (HL). The aim of this study was to confirm the prognostic impact of *HGAL* protein expression in an independent, well-characterized cohort of 232 patients with classic HL treated uniformly with doxorubicin, bleomycin, vinblastine and dacarbazine (ABVD). Tissue microarray analysis showed *HGAL* staining in 188 specimens (81%). Failure-free survival (FFS) was superior in patients with early-stage disease, low-risk IPS, and *HGAL*-positive patients. The estimated 5-year FFS for *HGAL*-positive and *HGAL*-negative patients was 82% and 67%, respectively ($p = 0.03$). In the multivariate analysis, advanced stage and absence of *HGAL* staining were independent predictors of a worse FFS. This study confirms and validates recent findings of a correlation between *HGAL* expression and outcome in classical HL.

Keywords

HGAL; Hodgkin lymphoma; survival; prognosis; tissue microarray

Introduction

The survival of patients with Hodgkin lymphoma (HL) has improved substantially over the last 40 years. In early-stage disease, the probability of cure reaches 90%, while in advanced

disease it ranges from 65 to 80% [1]. To further improve results, two different approaches have been pursued: to preemptively increase dose-intensity to all patients with advanced disease, or to try to identify beforehand those patients more likely to present with primary or secondary resistance to treatment [1].

Prognostic models centered on standard clinical and laboratory features were developed, but the identification of patients with poor prognosis remains a challenge [2]. It is presumed that these clinical features are surrogates for the intricate biological phenomena involved in the pathogenesis of the disease, and recent attempts have been made to pinpoint molecular features with prognostic relevance [3].

Data from gene expression studies have indicated that markers of germinal center (GC) derivation are associated with clinical behavior in diffuse large B-cell lymphoma (DLBCL) [4,5]. Using statistical methodology to supervise the exploration of gene expression profiling data, an expressed sequence tag that best predicted overall survival in DLBCL led to the cloning and characterization of the human germinal center-associated lymphoma (HGAL) gene [6]. It has nucleotide sequence homology to the mouse GC-specific gene M17, and was shown by hierarchical clustering to be contained within the GC gene cluster. Further work led to the development of a monoclonal antibody against HGAL and to the confirmation that HGAL protein expression is associated with other GC markers such as BCL6 and CD10 [7].

When tested in HLs, HGAL staining was found to be positive in 12 of 17 (71%) patients with lymphocyte-predominant HL, an expected finding due to the purported GC-origin of this disease. Interestingly, however, HGAL was also positive in 78 of 107 (73%) cases of classical Hodgkin lymphoma (cHL) [8]. The influence of HGAL protein expression on treatment outcomes in patients with cHL was previously tested in 145 patients and found to be correlated with better overall and failure-free survival (FFS) [8]. However, these differences did not remain significant in multivariate analyses that included well-known clinical factors, such as age and stage that impact the prognosis of patients with classical HL [8].

Confirmation of the predictive survival power of newly identified biomarkers is always needed in independent cohort of patients, preferentially treated at different institutions, before their usage in clinical trials and practice can be recommended. Consequently, the aim of this study was to confirm the previous findings regarding HGAL expression and prognostic impact in a large independent cohort of well-characterized Brazilian patients treated uniformly with the ABVD regimen. Correlation between HGAL and Epstein-Barr virus (EBV) expression was also explored to determine whether EBV is associated with HGAL-positive cHL cases.

Materials and methods

Patients' characteristics

This study included 232 consecutive cases of cHL treated on initial diagnosis at the University Hospital, Federal University of Rio de Janeiro and at the Brazilian Instituto Nacional de Cancer, from 1997 to 2004. Diagnoses were confirmed on review by three authors (DA, JCM, and YN) using morphologic and immunologic criteria defined in the World Health Organization (WHO) classification [9]. Patients were selected based on the availability of clinical information and histologic material for tissue microarray (TMA) construction. Expression of CD30 was required for inclusion. Patients with the acquired immune deficiency syndrome were excluded. All patients were staged according to the Ann Arbor system. The following baseline clinical characteristics were recorded: sex, age, stage, presence of bulky disease or B symptoms, performance status, and blood counts. The International Prognostic Score (IPS) was computed [2]; patients were categorized as low-risk IPS if they presented with up to two risk factors and

high-risk IPS if three or more risk factors were present. Patients were also stratified into early stage (I–IIA) and advanced (IIB–IV) cHL.

All patients were treated with curative intent. Patients with early stage disease were treated with two to four cycles of ABVD followed by radiation therapy. Patients with advanced disease were treated with six to eight cycles of ABVD, complemented by radiation therapy in patients with bulky disease. Patients who did not respond to primary therapy received either salvage chemotherapy or high-dose chemotherapy with autologous stem cell transplantation.

Tissue microarray construction and immunohistochemistry

Standard methods of tissue fixation (10% buffered formalin) and processing were used. TMAs were constructed using a tissue arrayer (Beecher Instruments, Silver Spring, MD) as previously described [10]. Duplicate 2 mm cores were obtained from each patient's paraffin block. Tissue cores from each case were selected by morphologic characteristics on hematoxylin and eosin stained original slides. Four-micrometer paraffin sections from TMA were cut and dried overnight at 56°C, dewaxed in xylene and rehydrated using serial concentrations of ethanol according to the standard procedures. Heat-induced antigen retrieval was performed by microwaving with citrate buffer (10 mM; pH 6; for 10 min). Endogenous peroxidase activity was blocked in 5% alcoholic hydrogen peroxide for 30 min. Primary antibody against HGAL (1:40 dilution) was used as previously described [8]. A tyramide amplification system was used (Dako Catalyzed Signal Amplification System, Dako, Carpinteria, CA). Detection was carried out using a modified avidin–biotin–diaminobenzidine method. The percentage of HGAL-positive Hodgkin and Reed–Sternberg (HRS) cells was assessed independently by two pathologists (DA and YN) and consensus was reached by subsequent joint-review over a multi-headed microscope. As previously described [8], the data were stratified according to the number of HRS cells in the TMA cores that stained for HGAL and designated 'strong' if 30% or more, 'weak' if 5–30% and 'negative' if <5% of the HRS cells stained for HGAL.

In situ hybridization was performed on TMA sections using the INFORM EBER probe (Ventana, Tucson, AZ). Slides were stained on an automatic immunostainer (Ventana Benchmark) using the Ventana ISH/VIEW kit. Specific nuclear signal was considered positive.

Statistical analysis

Comparison of clinical characteristics between patients with cHL with and without *HGAL* expression was performed by Fisher exact test (two-sided) for categorical variables. Overall survival was defined as the time interval between the date of diagnosis to the date of death or last follow-up. FFS was defined as the time interval between the date of initial diagnosis and the date of disease progression or death from any cause, whichever came first. Survival curves were estimated using the product-limit method of Kaplan–Meier and were compared using the log-rank test. The Statistical Package for the Social Sciences (SPSS) version 15.0 software (Chicago, IL) was used for data analysis. Variables achieving $p < 0.2$ in the univariate survival analysis were entered by forward stepwise selection in a multivariate Cox analysis. Variables were treated as binary variables and were entered into the model with critical entry level and removal of p values of 0.05 and 0.1, respectively. Hazard ratios (HR) with their 95% confidence intervals (CI) were computed. Two-tailed $p < 0.05$ was considered statistically significant.

Results

Patient characteristics and treatment results

Among the 232 patients available for study, median age was 29 years (range, 15–82 years) and 108 (46%) were women. The patients' main characteristics at diagnosis are shown in Table I. The distribution of morphologic subtypes of cHL included nodular sclerosis 68%, mixed

cellularity 20%, lymphocyte depletion 4%, and lymphocyte-rich 3%. There were 5% of cHL that could not be further classified into subtypes.

All patients were treated with curative intent: 46% received only ABVD chemotherapy, while 54% received a combination of ABVD with radiation therapy. A total of 207 patients (89%) achieved complete remission after initial treatment, and 21 relapsed. Other 19 patients failed primary therapy. Among the 40 patients with primary or secondary failure, 16 received salvage chemotherapy and 24 were treated with high-dose chemotherapy with autologous stem cell transplantation. The median follow-up of the whole cohort of patients was 6.2 years (range, 0.2–11.7 years).

Human germinal center-associated lymphoma staining identifies a subset of cHL with superior failure-free survival

HGAL protein was expressed in 188 specimens (81%) while 44 (19%) were HGAL-negative. Among HGAL-positive cases, strong staining was observed in 113 cases (60%), while the remaining 75 cases (40%) showed weak staining. HGAL staining was localized to the cytoplasm of Hodgkin cells in all biopsies. The mean number of HRS cells present in each core was 49 (± 37). Previous study evaluating HGAL in cHL demonstrated an excellent correlation between staining results obtained from evaluation of TMA cores or whole paraffin blocks' sections (8).

There were no associations between evaluated clinical characteristics and HGAL expression (Table I). There was no apparent difference in HGAL expression in the different histologic subtypes of cHL. Also, no relationship was found between the expression of HGAL and EBV.

The correlations between clinical prognostic factors, HGAL staining, and patients' survival are summarized in Table II. The complete remission rate for HGAL-positive patients was 91%, compared with 82% for HGAL-negative patients ($p = 0.1$).

The 5-year FFS of the whole cohort of 232 patients was 79% (CI 95%: 74–84%). FFS was better in patients with early-stage disease and IPS; there was a trend for an association with absence of B symptoms and good performance status. HGAL expression was correlated with FFS. The estimated 5-year FFS for HGAL-positive patients was 82% (CI 95%: 76–86%), compared with 67% (CI 95%: 53–81%) for HGAL-negative patients [$p = 0.03$, Figure 1(A)].

The 5-year overall survival of the whole cohort of 232 patients was 90% (CI 95%: 86–95%). The overall survival was better in patients with young age, early-stage, absence of B symptoms, low-risk IPS, and good performance status. The 5-year overall survival of HGAL-positive and HGAL-negative patients was 91% (CI 95%: 86–95%) and 88% (CI 95%: 78–98%), respectively [$p = 0.24$, Figure 1(B)].

Multivariate analyses of FFS were performed including HGAL and the clinical parameters are listed in Table II. Because stage is an integral part of the IPS, separate multivariate analyses were performed based on HGAL staining and IPS risk groups, or HGAL staining and stage, B symptoms and performance status. When HGAL and IPS are included in the model, IPS remained an independent predictor. When stage B symptoms and performance status were included along with HGAL, advanced stage and lack of HGAL were independent predictors of worse FFS ($p = 0.02$ in both cases), with HR of 2.1 (IC 95%: 1.12–3.9) and 2 (IC 95%: 1.08–3.8), respectively.

Discussion

In this study, we sought to validate the recent finding that the expression of HGAL at the time of diagnosis is related to outcome in patients with cHL. It is absolutely necessary to validate the prognostic power of each newly identified biomarker in an independent cohort of patients. In addition, we aimed to determine if HGAL can be combined with clinical factors to identify subgroups with particularly good or poor prognosis.

The selected cohort of patients had features similar to the general cHL population at presentation. Furthermore, the response to the ABVD therapy in this cohort is similar to previously reported results achieved with this therapeutic approach [1], thus indicating that the studied cohort is representative of the general patient population with cHL. Our findings demonstrate that HGAL expression is correlated with FFS, but not with overall survival in patients with cHL. The association of a positive HGAL stain with improved FFS remained significant in the multivariate analysis, along with stage, over-riding the effect of B symptoms and performance status. Since the prognosis of patients with HL is excellent with reported cure rates approaching 80–90%, defining HL subgroups with 15% difference in FFS is clinically relevant. These findings are concordant with our previous observations that HGAL expression predicts improved failure-free survival of patients with cHL, but failed to confirm HGAL's predictive power for overall survival. This discrepancy may be related to the different salvage approaches used in this study and previously reported cohorts of patients, since the overall survival accounts for the effects of different salvage regimens given to patients with primary resistance to treatment, and to those who relapsed after a complete remission. In contrast, FFS is a better indicator of response to initial therapy and its correlation with HGAL expression confirms that this biomarker can be useful for the identification of cHL subgroups with distinct response to the standard initial therapy.

The confirmation that HGAL, a protein highly specific to GC-derived lymphomas, is expressed in 70–80% of patients with cHL sheds light on the pathogenesis and derivation of this disease. Although HRS cells were found to be of B cell origin due to the presence of rearranged and somatically mutated Ig V genes, they show an immunophenotype that is different from any other cell in the hematopoietic system, with co-expression of markers unrelated or not specific to the B lineage since the B-lineage-specific gene expression program was shown to be lost in HRS cells [11]. Although IL-4- and IL-13-induced signaling may contribute to HGAL expression and regulation [8], its expression in cHL cells most likely represents its derivation from GC B-cells. Although the function of HGAL is largely unknown, recent studies revealed that it affects the motility of normal GC lymphocytes as well as lymphoma cells [12]. Whether HGAL may affect additional characteristics of malignant lymphocytes that could contribute to the improved response to therapy and better clinical outcome is currently under intense investigation.

In conclusion, this study validates the recent finding of a correlation between HGAL protein expression and patient outcome in a large, clinically well-characterized and uniformly treated representative cohort of patients with classical HL. HGAL retained its independent predictive power for FFS in the multivariate analysis, when individual components of the IPS were incorporated, but it was not an independent predictor when the IPS itself, composed of multiple clinical variables, was introduced in the analysis. While this finding indicates that HGAL expression may not have additional prognostic value over the IPS alone, the results of this study suggest that there may be underlying differences in the biology of cHL tumors. Consequently, identification of prognostic markers such as HGAL, along with other similarly validated markers, may not only improve our prognostic ability in cHL but may also elucidate the complex biology of these tumors.

Acknowledgments

We are indebted to Fernando Augusto Soares MD, PhD and to Carlos Ferreira do Nascimento from the Department of Pathology of Hospital do Cancer A. C. Camargo for the TMA construction.

Declaration of interest: DA was supported by a CAPES/PDEE grant; ISL is supported by National Institutes of Health (NIH, Bethesda, MD) CA109335, NIH CA 122105, and NIH U56 CA112973 and Dwoskin Family Foundation (Miami, FL); IB is supported by Faperj grant 171.243/2006; YN is supported by grant NIH POI CA34233; NS is supported by Faperj grant 102.900/2008, and CNPq grant 302708/2008-1.

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Figure 1. Survival according to HGAL protein expression. (A) Failure-free survival according to HGAL expression ($p < 0.03$). (B) Overall survival according to HGAL expression ($p = 0.24$).

Table 1

HGAL expression and clinical features of patients

Feature*	All patients, N = 232	HGAL negative, N = 44 (19%)	HGAL positive, N = 188 (81%)	p value
Age (years)				
<45	180 (78)	34 (77)	146 (78)	1
≥45	52 (22)	10 (23)	42 (22)	
IPS				
0-2	141 (72)	23 (66)	118 (74)	0.4
≥3	54 (28)	12 (34)	42 (26)	
Performance status				
0-1	209 (91)	38 (86)	171 (92)	0.2
≥2	20 (9)	6 (14)	14 (8)	
Ann Arbor stage				
I-IIA	98 (42)	20 (45)	78 (41)	0.7
IIB-IV	134 (58)	24 (55)	110 (59)	
Histology				
Nodular sclerosis	159 (68)	27 (61)	132 (70)	0.6
Mixed cellularity	47 (20)	11 (25)	36 (19)	
Lymphocyte rich	7 (3)	1 (2)	6 (3)	
Lymphocyte depletion	9 (4)	2 (5)	7 (4)	
Non-classified	10 (5)	3 (7)	7 (4)	
Bulky disease				
No	138 (59)	27 (61)	111 (59)	0.9
Yes	94 (41)	17 (39)	77 (41)	
B symptoms				
No	121 (52)	23 (52)	98 (52)	1
Yes	111 (48)	21 (48)	90 (48)	
Hemoglobin (g/dL)				
≥10.5	175 (78)	28 (68)	147 (80)	0.1
<510.5	50 (22)	13 (32)	37 (20)	
Albumin (g/dL)				
≥4	83 (50)	13 (43)	70 (51)	0.5

Feature*	All patients, N = 232	HGAL negative, N = 44 (19%)	HGAL positive, N = 188 (81%)	p value
<4	84 (50)	17 (57)	67 (49)	
Leucocytes (/mm ³)				
<5 × 10 ⁹ /L	194 (87)	38 (93)	156 (85)	0.3
≥15 × 10 ⁹ /L	30 (13)	3 (7)	27 (15)	
Lymphocytes				
≥0.6 × 10 ⁹ /L	162 (91)	31 (89)	131(91)	0.7
<0.6 × 10 ⁹ /L	17 (9)	4 (11)	13 (9)	
Epstein-Barr virus				
Positive	86 (41%)	18 (50%)	68 (40%)	0.27
Negative	122 (59%)	18 (50%)	104 (60%)	

HGAL, human germinal center-associated lymphoma; IPS, International Prognostic Score.

* Not all clinical features were available for every patient.

Table II

Patient and disease characteristics and univariate analysis

	Patients	Overall survival			Failure-free survival		
		No. of deaths	5-year OS%	p value	No. of events	5-year FFS%	p value
Sex				0.6			0.8
Male	124	13	89		25	80	
Female	108	9	92		23	78	
Age (years)				0.003			0.4
<45	180	12	94		35	81	
≥45	52	10	79		39	73	
Stage				0.01			0.03
I–IIA	98	4	97		14	85	
IIB–IV	134	18	86		34	74	
IPS				<0.0001			0.001
≤2	141	6	96		20	85	
>2	54	10	82		18	68	
Bulky				0.4			0.5
No	138	15	89		31	77	
Yes	94	7	93		17	81	
B symptoms				0.01			0.07
No	121	16	97		20	83	
Yes	111	6	84		28	74	
PS				<0.0001			0.07
0–1	209	16	92		41	80	
≥2	20	6	70		7	65	
HGAL				0.24			0.03
Positive	188	16	91		34	82	
Negative	44	6	88		14	67	

IPS, International Prognostic Score; PS, performance status; HGAL, human germinal center-associated lymphoma.