

LGALS3 as a prognostic factor for classical Hodgkin's lymphoma

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LGALS3, a member of the lectin family, has an important role in tumor progression through inhibition of apoptosis. LGALS3 shares several significant structural properties with BCL2. In this study, we examined the prognostic significance of LGALS3 and BCL2 in uniformly treated classical Hodgkin's lymphoma. Diagnostic tissues from 110 patients with uniformly treated classical Hodgkin's lymphoma were evaluated retrospectively by immunohistochemical analysis of LGALS3 and BCL2 expression. The median follow-up time was 6.2 years (range, 0.2–17.3 years). Twenty-seven patients (25%) expressed LGALS3 protein in Hodgkin/Reed–Sternberg cells, which was associated with poor overall survival and event-free survival ($P=0.007$ and $P<0.001$). Fifteen patients (14%) expressed BCL2 protein in Hodgkin/Reed–Sternberg cells, which was not associated with overall survival and event-free survival ($P=0.928$ and $P=0.900$). There was no correlation between LGALS3 and BCL2 expression ($P=0.193$). Multivariate analysis identified LGALS3 protein as an independent prognostic factor for event-free survival ($P=0.007$). Subgroup analysis according to the Ann Arbor stage of classical Hodgkin's lymphoma showed that LGALS3 protein expression had a prognostic value in limited-stage classical Hodgkin's lymphoma ($P<0.001$). The results of this study suggest that LGALS3 is an independent prognostic factor in classical Hodgkin's lymphoma, and may allow the identification of a subgroup of patients with limited-stage classical Hodgkin's lymphoma who require more intensive therapy.

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Classical Hodgkin's lymphoma is a highly curable lymphoid malignancy.¹ However, despite important treatment advances, a minority of patients do not respond to therapy or relapse after optimal initial therapeutic strategy, and may require adapted first-line treatment.² While the International Prognostic Score is useful in clinical practice, it does not fully reflect the biological behavior of classical Hodgkin's lymphoma.³

Galectins, a family of mammalian β -galactoside-binding lectin molecules, are implicated in inflammation and tumor progression.⁴ LGALS3 is expressed in lymphoma and myeloma cells,^{5,6} and has both pro-apoptotic and antiapoptotic functions.⁷ A recent study demonstrated that high

expression of LGALS3 protein in diffuse large B-cell lymphoma cells was associated with resistance to apoptosis.⁶ Moreover, LGALS3 has an important role in angiogenesis and metastases in several malignancies, including B-cell lymphomas.^{7–9}

B-cell lymphoma-2 (BCL2) protein inhibits apoptosis by preventing cytochrome release from mitochondria, thus blocking oxidative cell destruction. Over-expression of BCL2 has been found in some solid malignancies and malignant lymphomas including classical Hodgkin's lymphoma.^{10–13} The BCL2 family of antiapoptotic proteins contribute to the survival of Hodgkin/Reed–Sternberg cells by counteracting the expression of the pro-apoptotic proteins bax, and bad.¹³ There is specific sequence homology between the NWGR (Asp–Trp–Gly–Arg) amino acid sequence in *LGALS3* and the BH1 domain of the *BCL2* gene family.¹⁴ Furthermore, LGALS3/BCL2 heterodimerization promotes the BCL2 antiapoptotic effect.¹⁴

Although there is evidence that LGALS3 and BCL2 are associated with tumor progression in

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hematologic malignancies including B-cell lymphomas,^{14,15} no study has examined the relationship between LGALS3 and BCL2, and their prognostic implication in classical Hodgkin's lymphoma patients. This retrospective study evaluated LGALS3 and BCL2 expression in classical Hodgkin's lymphoma patients and examined the correlations between these markers and their prognostic significance.

Materials and methods

Patients

This retrospective study reviewed histological and immunohistochemical data from 110 consecutive patients diagnosed with classical Hodgkin's lymphoma at Asan Medical Center, Seoul, South Korea, between 1990 and 2012. All patients had pathologically confirmed classical Hodgkin's lymphoma, treated with doxorubicin, bleomycin, vinblastine, and dacarbazine therapy regimen, with or without radiation. The patients were aged ≥ 15 years at diagnosis, and had no previous treatment and no history of malignancy. All samples were obtained before to treatment. Follow-up data were available for all patients included in the study.

The median follow-up time was 6.2 years (range, 0.2–17.3 years). Response criteria were based on standard guidelines. Routine follow-up imaging analyses were performed every 3 months for the first 2 years, every 6 months for the next 3 years, and annually (or whenever clinically indicated) thereafter. We defined the limited-stage as Ann Arbor stage I–II without B symptom or bulky disease.

The present research was approved by the Internal Review Board of the Asan Medical Center.

Histopathological Analysis and Immunohistochemistry

All histological and immunophenotypic data were reviewed by three pathologists (JH, YWK, and SJJ). According to the World Health Organization criteria, the classical Hodgkin's lymphoma cases were subtyped as follows: nodular sclerosis, lymphocyte-rich, mixed cellularity, lymphocyte-depleted, or not otherwise specified classical Hodgkin's lymphoma.¹⁶ Tissue microarray was constructed with three 1 mm diameter tumor cores from selected areas of formalin-fixed, paraffin-embedded tumor samples. The tissue microarray section was stained using an automatic immunohistochemistry staining device (Benchmark XT, Ventana Medical System, Tucson, AZ, USA). Briefly, 5 μ m thick sections were transferred onto poly-L-lysine-coated adhesive slides and dried at 62 °C for 30 min. After standard heat epitope retrieval for 30 min in ethylenediamine tetraacetic acid (pH 8.0), the samples were incubated with antibodies against cleaved LGALS3 (dilution 1:200; NOVO, Newcastle, UK), and BCL2 (dilution 1:50; Cell Marque, Rocklin, CA, USA). The sections were then incubated with biotinylated antimouse immunoglobulins, peroxidase-conjugated streptavidin (LSAB kit, DAKO, Glostrup, Denmark), and 3,3'-diaminobenzidine. Slides were counterstained with Harris hematoxylin.

In the tissue microarray, each case was represented by three tissue cores, and at least ten CD30-positive Hodgkin/Reed–Sternberg cells, in at least one of the three core cylinders from each patient, were read. A sample was considered LGALS3- or BCL2-positive if the expression of these markers was detected in $\geq 10\%$ of Hodgkin/Reed–Sternberg cells (Figures 1a and b). A sample was considered LGALS3- or BCL2-negative if LGALS3 or BCL2 expression was detected in $<10\%$ of the Hodgkin/Reed–Sternberg cells, or in bystander cells only.

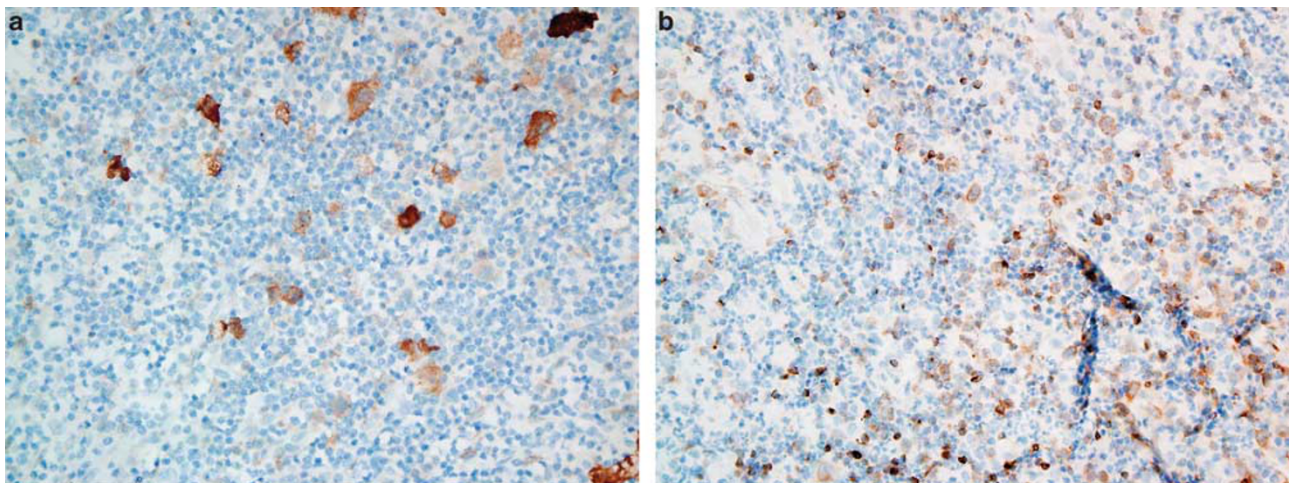


Figure 1 LGALS3 and BCL2 expression in classical Hodgkin's lymphoma tissues. Hodgkin/Reed–Sternberg cells showed strong LGALS3 immunopositivity in the cytoplasm (a; original magnification, $\times 400$) and BCL2 immunopositivity in the membrane (b; original magnification, $\times 400$).

In situ hybridization analysis of Epstein-Barr Virus (EBV)-encoded RNA-1 and RNA-2 was performed and scored as previously described.¹⁷

Statistical Analysis

Overall survival was defined as the interval between the date of diagnosis and death from any cause. The follow-up of living patients was censored at their last follow-up date. Event-free survival was defined as the interval between the date of diagnosis and the date of disease progression, relapse, or death from any cause. Cumulative overall survival and event-free survival were analyzed by the Kaplan–Meier method, and comparisons were performed by log-rank testing.

Multivariate prognostic analyses of overall survival and event-free survival were performed with the Cox proportional hazards regression model using the enter method. Categorical variables were compared using the χ^2 -test. All statistical analyses were performed using the SPSS statistical software program (version 18.0; SPSS, Chicago, IL, USA) or R 2.15.2. All *P*-values are two-sided associations and *P* < 0.05 was considered statistically significant.

Results

Patient Characteristics

The clinical characteristics of the 110 patients included in the study are summarized in Table 1. The median age of the patients was 35 years (range, 15–77 years). Forty-two patients experienced relapse, disease progression, or death, and 20 patients died. Median overall survival and event-free survival were not reached. The estimated 5-year overall survival and event-free survival were 83 and 58%, respectively.

LGALS3 and BCL2 Expression in Classical Hodgkin's Lymphoma Tissues

The correlations between LGALS3, BCL2, and clinical variables are summarized in Supplementary Table 1. The expression of LGALS3 or BCL2 was not associated with clinical variables. There was no correlation between LGALS3 and EBV status (*P* > 0.999) and between BCL2 and EBV status (*P* = 0.162). There was no correlation between LGALS3 and BCL2 expression (*P* = 0.193).

Prognostic Significance of LGALS3 and BCL2 Expression

Patients with LGALS3 expression had lower 5-year overall-survival rates (73 vs 88%, *P* = 0.007; Figure 2a), and lower 5-year event-free survival rates (44 vs 67%, *P* < 0.001; Figure 2b) than patients

Table 1 Demographic and clinical characteristics of patients

Characteristics at diagnosis	Number of patients (%)
Age, median (range, years)	35 (15–77)
Male gender	64 (58)
<i>Histologic subtype</i>	
Nodular sclerosis	74 (67)
Mixed cellularity	22 (20)
Lymphocyte-rich	5 (5)
Lymphocyte-depleted	3 (3)
Not classifiable	6 (6)
<i>Ann Arbor stage</i>	
I	21 (19)
II	37 (34)
III	26 (24)
IV	26 (24)
<i>Stage (limited vs advanced)</i>	
Limited	41 (37)
Advanced	69 (63)
B symptoms present	35 (32)
International Prognostic Score ≥ 4 (high-risk)	20 (18)
Epstein-Barr virus-encoded RNA positivity	41 (37)
<i>Primary treatment</i>	
Chemotherapy	81 (74)
Chemoradiotherapy	29 (26)

with no LGALS3 expression. BCL2 expression was not significantly associated with either overall survival or event-free survival (*P* = 0.928, Figure 2c; and *P* = 0.900, Figure 2d, respectively).

Univariate analysis revealed that both overall survival and event-free survival were associated with the International Prognostic Score (≥ 4). Multivariate analysis identified LGALS3 index as an independent prognostic marker for event-free survival (*P* = 0.007, Table 2), along with high-risk International Prognostic Score (≥ 4), and a marginal prognostic marker for overall survival (*P* = 0.063, Table 2).

As the International Prognostic Score is a significant prognostic factor only in advanced-stage disease,³ we performed a subgroup analysis according to the disease stage, to determine whether LGALS3 may have an advantage over the International Prognostic Score. In limited-stage classical Hodgkin's lymphoma cases, high LGALS3 expression was associated with worse overall-survival rate compared with low LGALS3 expression, (*P* < 0.001; Figure 3a). In patients with advanced-stage classical Hodgkin's lymphoma, LGALS3 positivity was not associated with overall survival (*P* = 0.189; Figure 3b).

By contrast, the International Prognostic Score was not significantly associated with overall survival in limited-stage classical Hodgkin's lymphoma (*P* = 0.468; Figure 3c), although, it was significantly associated with overall survival in advanced-stage

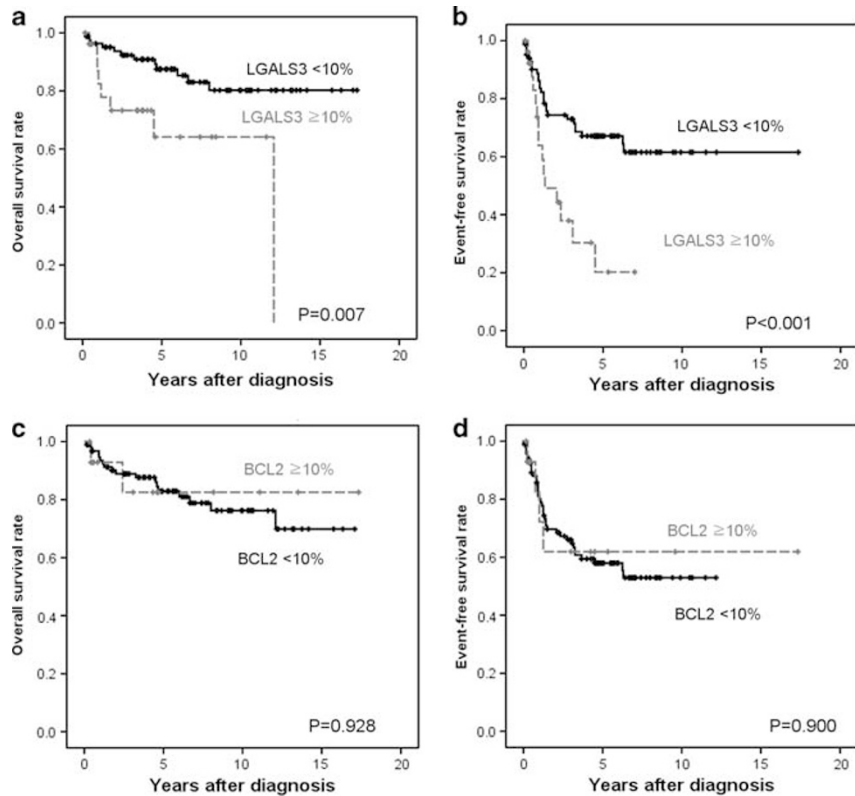


Figure 2 Comparison of survival rates according to LGALS3 and BCL2 expression. Overall survival (a) and event-free survival (b) were significantly worse in the LGALS3-positive cases. BCL2 expression was not significantly associated with either overall survival (c) or event-free survival (d).

Table 2 Univariate analysis of overall survival and event-free survival

		Univariate analysis					
		Overall survival			Event-free survival		
Covariate		HR	95% CI	P-value ^a	HR	95% CI	P-value ^a
Age	<45 vs ≥45	6.001	2.17–16.5	<0.001	2.606	1.41–4.79	0.002
Sex	Female vs male	2.832	1.02–7.82	0.045	1.690	0.89–3.18	0.104
B symptoms	(–) vs (+)	1.797	0.74–4.36	0.195	1.265	0.67–2.37	0.466
International Prognostic Score	<4 vs ≥4	4.796	1.94–11.8	<0.001	2.958	1.50–5.82	0.002
LDH (U/L)	Normal vs abnormal	1.401	0.49–3.95	0.524	1.070	0.56–2.01	0.835
EBER	(–) vs (+)	1.619	0.66–3.92	0.286	1.615	0.87–2.96	0.122
LGALS3 expression	(–) vs (+)	3.253	1.30–8.08	0.011	2.919	1.52–5.57	<0.001
Treatment	Chemotherapy vs chemoradiotherapy	0.513	0.17–1.53	0.234	0.633	0.31–1.28	0.207

		Multivariate analysis					
		Overall survival			Event-free survival		
Covariate		HR	95% CI	P-value	HR	95% CI	P-value
International Prognostic Score	<4 vs ≥4	3.977	1.56–10.1	0.004	2.458	1.22–4.93	0.011
LGALS3 expression	(–) vs (+)	2.432	0.95–6.21	0.063	2.512	1.28–4.89	0.007

Abbreviations: CI, confidence interval; HR, hazard ratio; EBER, Epstein-Barr virus-encoded RNA-1 and RNA-2 assessed by *in situ* hybridization; LDH, lactate dehydrogenase.

^aCox univariate analysis.

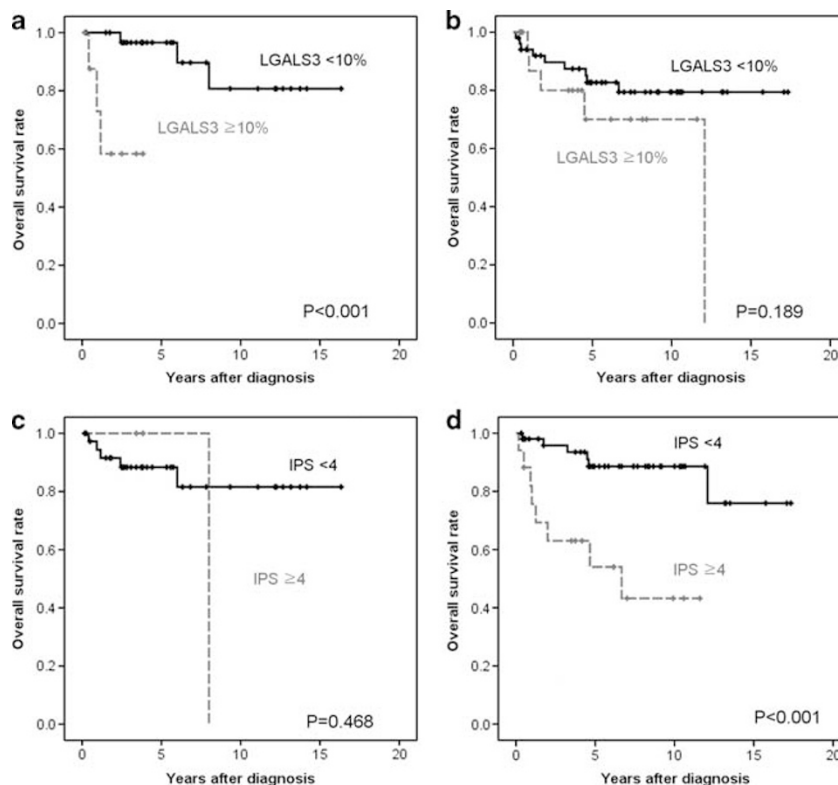


Figure 3 Comparison of survival rates according to LGALS3 expression and Ann Arbor stage of classical Hodgkin's lymphoma. LGALS3 positivity in patients with limited-stage classical Hodgkin's lymphoma was associated with worse overall survival (a). In patients with advanced-stage classical Hodgkin's lymphoma, LGALS3 positivity was not associated with overall survival (b). Comparison of survival rates according to International Prognostic Score and Ann Arbor stage. Patients with high-risk International Prognostic Score was not associated with worse overall survival (c) in patients with limited-stage classical Hodgkin's lymphoma, however, high-risk International Prognostic Score was associated with overall survival (d) in advanced-stage classical Hodgkin's lymphoma.

classical Hodgkin's lymphoma cases ($P < 0.001$; Figure 3d).

Discussion

This study is the first to evaluate the prognostic value of LGALS3 expression in classical Hodgkin's lymphoma patients. In a cohort of 110 patients with ABVD-treated classical Hodgkin's lymphoma, LGALS3 expression had a prognostic value in limited-stage classical Hodgkin's lymphoma. However, BCL2 expression was not associated with LGALS3 expression and had no prognostic value.

Intracellular LGALS3 protects different cell types against apoptosis in response to various stimuli.⁷ Thus, LGALS3-transfected Jurkat cells become resistant to apoptosis induced by anti-Fas antibody and staurosporine, compared with controls.¹⁸ Likewise, Burkitt lymphoma cells transfected with a *LGALS3*-expressing plasmid show increased resistance to anti-Fas-induced cell death.⁸ On the other hand, secreted extracellular LGALS3 molecules induce T-cell apoptosis, thereby having an important role in the immune escape during tumor progression, through induction of apoptosis of cancer-infiltrating T-cells.¹⁹ We found that

intracellular LGALS3 was associated with poor prognosis in classical Hodgkin's lymphoma patients, in agreement with previous studies of LGALS3 in solid tumors and other lymphomas.^{7,8,18}

In contrast to LGALS3, BCL2 expression was not associated with overall survival or event-free survival. Although LGALS3 is not a member of the BCL2 gene family, it shares several significant structural properties with BCL2.¹⁴ The antiapoptotic activity of LGALS3 is mediated, at least in part, by the activation of ERK and JNK (c-Jun NH₂-terminal kinase) pathway²⁰ or CD95 (APO-1/Fas) receptor, a member of the death receptor family.²¹ Further studies are needed to determine the precise mechanism of LGALS3-mediated antiapoptosis in classical Hodgkin's lymphoma patients.

In addition to its antiapoptotic effect, LGALS3 has a role in cell growth, cell adhesion, cell differentiation, and tumor progression and metastasis, mainly through binding to glycoproteins.^{22–24} LGALS3 stimulates capillary tube formation by human umbilical vein endothelial cells *in vitro*, and angiogenesis *in vivo*.²⁵ Furthermore, LGALS3 increases $\alpha 4$ and $\beta 7$ integrin expression in the breast cancer cell line Evsa-T and promotes Evsa-T adhesion to laminin, fibronectin, and vitronectin,²⁶ which suggests that LGALS3 may induce metastasis

by specifically influencing cell adhesion to the extracellular matrix. Further studies are needed to delineate the mechanism of LGALS3-mediated adverse effects on the prognosis of classical Hodgkin's lymphoma patients.

EBV, a ubiquitous herpes virus that infects more than 90% of humans, is now classified as a group 1 human carcinogen by the International Agency for Research on Cancer owing to its etiologic role in classical Hodgkin's lymphoma.²⁷ Our study found no correlation between LGALS3 expression and EBV status. These results suggest that LGALS3 expression was associated with survival outcome independently of EBV status.

In the present study, LGALS3 expression was significantly associated with poor prognosis, especially for patients with limited-stage disease. Although the International Prognostic Score is the standard stratification system for survival in patients with advanced-stage disease, it fails to stratify the subgroups with worse prognosis in patients with limited-stage classical Hodgkin's lymphoma.³ Our results also revealed that the International Prognostic Score was associated with prognosis only in advanced-stage classical Hodgkin's lymphoma. Therefore, LGALS3 expression is a viable prognostic factor alternative to the International Prognostic Score in limited-stage classical Hodgkin's lymphoma.

Studies assessing the prognostic significance of BCL2 expression in patients with classical Hodgkin's lymphoma have yielded conflicting results. Although several reports found a significantly negative prognostic effect of its over-expression in Hodgkin/Reed–Sternberg cells,^{28–33} other studies found no impact.^{34–36} Possible reasons for the inter-study discrepancy include disparate study populations, technical differences, for example, different antibody clones, use of tissue microarray vs whole sections, inter-observer variability, different criteria of BCL2 positivity, and disparate use of the index of outcome. For example, Sanchez-Espiridon *et al*³³ used a population with advanced-stage patients, whereas we studied a series of consecutive patients in one hospital. While the chemotherapy regimens in some previous studies were varied,^{29,30} we limited our analysis to patients treated with a doxorubicin, bleomycin, vinblastine, and dacarbazine regimen.

The limitations of this study include its retrospective design, short follow-up period of some recent cases, small sample size, and tissue microarray-based design. Tissue microarray design cannot reflect the entire distribution of tumor because of tissue heterogeneity.

In summary, our results suggest that LGALS3 is an independent prognostic factor in classical Hodgkin's lymphoma, and may be useful in the identification of a subgroup of patients with limited-stage classical Hodgkin's lymphoma who are at a high risk for recurrence or progression and who may benefit from

aggressive chemotherapy. Further studies, including prospective clinical trials, are needed to confirm the present findings, and investigate the effects of LGALS3 expression on clinical outcomes.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (<http://www.nature.com/modpathol>)