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Clinical relevance of galectin-1 expression in non-small cell lung cancer patients

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Abstract

BACKGROUND: Identification of biomarkers in lung cancer, a leading cause of cancer-related mortality, has a meaningful clinical relevance in the quest of novel prognostic factors and therapeutic targets. The glycan-binding protein galectin-1 (Gal-1) modulates tumor progression by mediating cell-cell and cell-extracellular matrix interactions, as well as angiogenesis and tumor immune-escape. Previous works reported the expression of Gal-1 in lung cancer, although its clinical significance remains uncertain. OBJECTIVE: To assess the clinicopathologic relevance and prognostic value of Gal-1 expression in a cohort of 103 Stage I-III non-small cell lung cancer (NSCLC) patients. METHODS: Gal-1 expression was determined by immunohistochemistry in tumor tissue samples. The percentage of immunoreactive tumor cells and stroma, as well as the presence of blood vessels with positively stained endothelium in the tumor and surrounding normal tissue, were recorded. Results were correlated with the clinicopathologic factors of the patients (Spearman's rank correlation coefficient, chi-square test) and overall survival by univariate (Kaplan Meier) and multivariate analyses (Cox regression hazard model). RESULTS: We did not observe significant associations between Gal-1 expression and relevant clinicopathologic features at diagnosis of NSCLC. However, Kaplan Meier analysis revealed a significant association between Gal-1 expression and overall survival, when Gal-1 expression was analyzed on tumor cells alone ("tumor cell percentage") or when an integrated score accounting for tumor cell as well as stromal expression of Gal-1 ("total score") was assessed. Patients showing high Gal-1 expression evidenced a poorer clinical outcome. Furthermore, "total score" remained significantly associated with survival by multivariate Cox regression analysis in the whole cohort of patients, even when controlling

for the classical predictors and prognostic factors of NSCLC. CONCLUSION: We conclude that Gal-1 expression may be a useful biomarker for better prediction of the clinical outcome and management of NSCLC patients.

Introduction

Lung cancer is the most frequent and one of the most deadly cancer types. In Argentina, the age-standardized mortality rates were 31.9 among men and 7.5 among women per 100000 inhabitants per year (1990 to 2005), being the leading cause of cancer-related death in men and the fourth leading cause in women [1]. Most lung cancers (85%) are NSCLC, which consist of squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. Treatment options depend on stage of disease and include surgery, radiation, platinum doublet chemotherapy, and targeted therapies in some cases [2]. The most important prognostic factor for survival is the tumor, node, metastasis (TNM) system, which has been revised in 2009 [3]. Nevertheless, data are still missing with regards to survival and predictive features, as even among patients at early stages of the disease, about 30% relapse and die within 5 years of surgery [4]. A better understanding of the mechanisms leading to adverse clinical outcomes is likely to improve therapeutic intervention of NSCLC patients.

Anti-tumor immune responses play important roles in eradicating and suppressing the growth of several cancer types. However, tumors can evade immune surveillance through a number of immune escape mechanisms and immunosuppressive molecules [5], some of which have served as clinical biomarkers delineating the prognosis of cancer patients (reviewed in [6]). Gal-1 belongs to a family of soluble lectins defined by a common structural fold and a conserved carbohydrate recognition domain that recognizes glycans containing the disaccharide N-acetyllactosamine [7]. Secretion of Gal-1 has been shown to contribute to the immunosuppressive activity of melanoma, Hodgkin's lymphoma and pancreatic carcinoma, suggesting the essential role of this protein in tumor-immune escape by tumors [8-10]. In lung cancer, several preclinical studies documented a link between

Gal-1, immune escape and tumor progression [11, 12]. Furthermore, a prognostic significance of Gal-1 in human lung cancer has been proposed [13, 14].

We undertook this study to provide a detailed retrospective assessment of Gal-1 expression in 103 cases of primary NSCLC stages I-III and analyzed its clinicopathologic role and prognostic significance. Our results indicate that an integrated score accounting for tumor as well as stromal expression of Gal-1 represents an independent prognostic factor of poor outcome in NSCLC patients.

Materials and methods

Patients and tumor specimens

103 samples from patients with NSCLC were obtained from the "Hospital Italiano de Buenos Aires" for this retrospective analysis. Tissue specimens were obtained at surgery from untreated patients, fixed in 4% (v/v) formaldehyde in PBS immediately after removal and embedded in paraffin. The study included white individuals Curative resections were performed from 1997 through 2009. Staging was determined by the American Joint Committee on Cancer Guidelines, 7th edition [3]. Data on pathological findings and clinical follow-up were obtained from medical charts. All patients who died had clear evidence of uncontrolled tumor growth at the time of death. The Ethics Committees of the Institute of Oncology "Ángel H. Roffo" and the "Hospital Italiano de Buenos Aires" approved this study, which carefully followed the Declaration of Helsinki. The requirement of informed consent was waived as the study was based in the retrospective analyses of existing administrative and clinical data. Table 1 summarizes the clinical and pathological features of all cases.

Immunohistochemistry

Five μm-tissue sections were obtained from each tumor block on polylysine-coated slides. All sections were routinely deparaffinized and rehydrated before staining. Sections were treated with 0.3% hydrogen peroxide in water for 30 min to block endogenous peroxidase activity. Immunohistochemical staining of Gal-1 was performed using a streptavidin-biotin amplification method with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame,

CA). Sections were incubated with normal horse serum in PBS for 45 min to block non-specific binding before adding the affinity-purified rabbit anti-Gal-1 primary antibody (diluted 1:2000 in PBS) overnight at 4°C. After washing, secondary biotinylated universal antibody was added for 1 h at room temperature followed by Vectastain ABC reagent for 30 min, washing between steps. The antigen-antibody complex was visualized with diaminobenzidine (Vector Laboratories, Burlingame, CA), counterstained with Mayer's hematoxylin, rehydrated, and then mounted with Canada synthetic balsam. Negative controls missing the primary antibody showed no immunoreactivity. The consistent expression of Gal-1 in peripheral nerves was used as an internal positive control.

Quantification of Gal-1 expression

Sections were coded and analyzed by two experienced observers without knowledge of patient evolution. The expression of Gal-1 was evaluated in the tumor cells and in the tumor stroma (cells and extracellular matrix) and categorized according to the **percentage of positive stained cells** (score from 0 to 3, where 0 = negative, 1 ≤ 10%, 2 = 11–50%, and 3 ≥ 51%) and the **intensity of staining** (0= negative, 1= weak, 2= moderate and 3= strong). By means of these percentages, patients were divided into two groups showing low or high Gal-1 expression by using 0% and 50% as cut-off values for tumor cells and tumor stroma, respectively. On this basis, “tumor cell percentage” and “tumor stroma percentage” were recorded. A combined score was also calculated for each tumor with a double-entry table combining the percentage and intensity values, establishing the variables “tumor cell score” and “tumor stroma score” (Table 2). Score data were dichotomized into two groups of low and high Gal-1 score using a cut-off value of 2. Also, an overall score (total score) integrating the dichotomized percentages of both tumor cells and stromal cells was

calculated and dichotomized establishing the high Total Score group when percentage was high for both tumor cells and stroma. Endothelial cells delineating blood vessels were also analyzed for Gal-1 immunoreactivity. Cases were segregated into a low or high expression group using a cut-off of 10% of immunoreactive endothelial cells in both tumor and surrounding normal tissue (tumor vessels, normal vessels).

Statistical analyses

For statistical purposes, all variables were dichotomized into a low-expression group and a high-expression group. Statistical correlations between the variables under study were assessed with Spearman's rank correlation coefficient. Dependence among the variables and the clinicopathologic parameters was evaluated by $\chi 2$ test. Global survival was measured from the date of surgery to the time of the last follow-up visit or death. Survival curves were plotted according to the Kaplan-Meier method and the *P* value of the likelihood-ratio test was used to assess statistical significance of the hazard ratios. To identify independent prognostic factors, we used the Cox proportional hazard model. *P* values are based on 2-sided tests, and significance was set at $P \leq .05$.

Results

Gal-1 Expression in Human NSCLC

Gal-1 was assessed by immunohistochemistry in 103 tissue specimens derived from primary NSCLC. Tumor cells showed positive diffuse or granular staining for Gal-1 that was exclusively cytoplasmic (Fig. 1a). Gal-1 expression was detected in 53/103 (51.5%) NSCLC samples ("tumor cell percentage"). The tumor stroma displayed widespread Gal-1 staining both in stromal cells and the extracellular milieu (Fig. 1c), with 85/103 (82.5%) immunoreactive cases in more than 50% of total tumor stroma ("tumor stroma percentage"). Finally, a score integrating positivity of both tumor cells and stroma was calculated ("total score"). In addition, we assessed Gal-1 staining in endothelial cells to analyze a possible correlation with angiogenesis, both at the tumor (Fig. 1e,f) and normal tissue (Fig. 1g,h) (tumor vessels and normal vessels). Notably, 58/102 (56.9%) and 44/90 (48.9%) cases showed Gal-1 expression in vessels associated to tumoral and normal tissue, respectively. Remarkably, a strong correlation was observed between Gal-1 expression in vessels of tumoral and normal tissue (Spearman's rank correlation, R: 0.741, P <0.0001).

Gal-1 Expression and Clinicopathologic Features in Human NSCLC

Possible associations between the variables under study and clinicopathologic parameters (T, N, stage, age, sex and histologic type) were analyzed by chi square test. No significant associations were observed, except for a higher proportion of cases with high Gal-1 expression in tumor stroma ("tumor stroma score") in adenocarcinomas versus squamous cell carcinomas (93.4% versus 75%, P =0.018).

To gain further insights into the clinical relevance of Gal-1 expression in NSCLC, we analyzed the possible correlation between Gal-1 expression and clinicopathologic features (Table 3). No significant correlation was observed with regards to T, N, stage or age, except for a weak correlation between tumor cell score and age (Table 3).

Gal-1 Expression and Survival of Human NSCLC

In our cohort of NSCLC patients the 5-year overall survival rate was 86% for Stage Ia, 80% for stage Ib, 73% for Stage IIa, 33% for Stage IIb and 9% for Stage III, and this classification predicted survival reliably ($P = 5.88E^{-11}$). We examined whether dichotomized variables of Gal-1 expression could also predict disease-specific. No significant association was found between different variables of Gal-1 expression and disease-free survival (data not shown). On the other hand, univariate analysis for overall survival indicated that "tumor stroma percentage", "tumor stroma score", "tumor vessels" and "normal vessels" were found to be not associated with overall survival. However, when the variables "tumor cell percentage" and "total score" were analyzed, we could discriminate patients into low and high risk groups with statistical significance ($P = 0.029$ and $P = 0.033$, respectively). Figures 2a and 2b illustrate the Kaplan-Meier plots of overall survival curves showing the low and high Gal-1 expression groups. High Gal-1 expression was associated with decreased 5-year survival when analyzed according to "tumor cell percentage" and "total score" parameters. On the other hand, the overall survival curves for the combined percentage and intensity values of Gal-1 immunostaining in tumor cells ("tumor cell score") were similar to those shown by the "tumor cell percentage", indicating that these two variables do not have significant independent outcomes (data not shown). Importantly, by multivariate Cox

regression analysis, the "total score" remained significantly associated with survival (P = 0.047; Table 4) in the whole cohort of patients, even when controlling for the classical predictors and prognostic factors such as T, N, histologic type (which reliably predicted survival) and sex, whereas the "tumor cell percentage" exhibited a borderline significance in this analysis (P = 0.084, data not shown).

Finally, we performed an additional multivariate analysis by creating a decision tree to assess the effect of specific variables on survival, including known predictors of NSCLC prognosis (stage, histologic type, age, sex) and the variables of Gal-1 expression which were found to be significant by univariate analysis ("tumor cell percentage" or "total score"). In two separated tree-structured models, "tumor cell percentage" and "total score" differentiated survival to the best and were allocated to node I. Patients with the worse clinical outcome (high "tumor cell percentage" or "total score") were further dichotomized by stage in the second node (data not shown).

Discussion

In this retrospective study, we evaluated Gal-1 expression in tumor cells and stroma of human NSCLC samples to assess whether this information could offer any prognostic value for the management of this cancer type. We report an association between increased Gal-1 expression and decreased overall survival in a cohort of 103 NSCLC patients, after adjustment for other predictors of outcome. This finding may be relevant to guide improved medical treatment and, together with several lines of preclinical studies, our study establishes the rationale for targeting Gal-1 expression and/or disrupting its binding to specific glycans in human NSCLC therapy.

Expression of Gal-1 has been shown to be associated with disease progression and/or overall survival by univariate analysis in gastric cancer [15], cervical cancer [16] and pancreatic ductal adenocarcinoma [17]. However, only few studies have demonstrated that Gal-1 could be an independent prognostic factor by conducting multivariate analysis [18, 19]. Regarding lung cancer, in tissue microarrays from patients with lung adenocarcinoma, Gal-1 expression scores were higher for stage III compared with stage I or II patients [20]. Here we could observe no significant associations between tumor or stromal Gal-1 expression and the most relevant clinicopathologic features of NSCLC at diagnosis. However, we did observe a positive correlation between high Gal-1 expression and decreased 5-year survival when the "tumor cell percentage" and the "total score" for Gal-1 expression were analyzed. In a previous study, Szöke *et al* showed poorer prognosis in NSCLC and SCLC lung tumors showing Gal-1 expression [13]. The authors also evaluated Gal-3 expression and binding activities in lung cancer patients and demonstrated that Gal-3 negative cases displayed improved survival rate [13, 14].

Gal-1 is secreted to the extracellular milieu through a still poorly understood unconventional pathway and interacts with cell surface glycosylated ligands to modulate immune escape, angiogenesis and tumor cell migration [21]. However, Gal-1 can be also found in the cytoplasmic and nuclear compartments and plays intracellular roles by modulating signaling pathways leading to tumorigenesis via protein-protein interactions with the cytoplasmic H-RAS oncogene [22]. We found that about 50% of NSCLC samples exhibited specific intracellular Gal-1 staining within the cytoplasmic compartment of tumor cells. Remarkably, Gal-1 plays multiple roles in lung cancer that lead to tumor progression and metastasis. In human NSCLC cell lines, endogenous Gal-1 expression contributes to cell migration, invasion, anchorage-independent growth and resistance to cisplatin *in vitro* and to tumor growth *in vivo,* through mechanisms dependent on activation of p38, ERK1/2 and NF-κB signaling pathways and cyclooxygenase-2 up-regulation [20]. Interestingly, while intracellular Gal-1 signaling appeared to be important for promoting lung cancer progression, NSCLC tumor cells secrete low levels of Gal-1 and were non-responsive to treatment with exogenous Gal-1 [17]. Also, in oral squamous cell carcinoma and lung adenocarcinoma cell lines Gal-1 expression correlated with invasiveness [23]. Furthermore, Gal-1 has been shown to promote lung cancer metastasis by amplifying integrin $\alpha_6\beta_4$ and Notch1/Jagged2 signaling pathways [24].

In addition to the activities displayed by Gal-1 on cancer cells themselves, Gal-1 also contributes to tumor progression by shaping the tumor microenvironment through its ability to promote tumor-immune escape [25] and modulate angiogenic sprouting [26]. Silencing Gal-1 expression in the 4T1 breast tumor model induced a marked reduction of both tumor growth and the number of lung metastases [27]. This effect was abrogated when

mice were inoculated with wild-type 4T1 tumor cells in their contralateral flank, suggesting involvement of a systemic modulation of the immune response [18]. Moreover, Gal-1 attenuation in 4T1 cells also reduced the frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells within the tumor, draining lymph nodes, spleen and lung metastases [18]. In human and mouse lung tumors, tumor-derived Gal-1 has been reported to be a critical mediator that instructs dendritic cells to express and release heparin-binding epidermal growth factor, which, in turn, promotes tumor progression [11]. In addition, in the Lewis lung carcinoma (LLC) model, tumor derived-Gal-1 induces tumor growth and metastasis through impairment of T cell mediated immune responses [12]. Moreover, in Kaposi's sarcoma, a highly vascular tumor, we found that targeted disruption of Gal-1-N-glycan interactions eliminated hypoxia-driven angiogenesis and suppressed tumorigenesis [26]. In addition, in prostate cancer targeting Gal-1 abrogated neovascularization-related processes [28]. Furthermore, targeting Gal-1 in melanoma cells controlled both vascular and immunosuppressive programs [9, 29, 30]. Altogether, these preclinical observations highlight the relevance of Gal-1 as an emerging therapeutic target in a number of cancers. However, the lack of more definitive clinical data precluded the translation of these findings to clinical settings. Our results in NSCLC patients support the notion that Gal-1 expression in lung cancer is associated with disease progression and unfavorable prognosis.

Gal-1 expression has been reported to be either restricted to the tumor stroma [15, 16] or found in both tumor cells and stroma [15, 18, 20, 31]. Here we showed that in NSCLC Gal-1 is widely expressed in the tumor stroma. Indeed, with the exception of two cases, all samples of NSCLC that were negative for Gal-1 in tumor cells (about 50%) presented positive stromal staining. This group of patients that showed only stromal

staining evidenced a better survival compared with the remaining set of patients that expressed Gal-1 in both tumor cells and the stroma (data not shown). This observation suggests that the expression of tumoral Gal-1 is a key prognostic factor which determines the evolution of the disease. The importance of host versus tumoral Gal-1 was previously analyzed in LLC tumor progression. Using different experimental strategies, the authors found that tumoral Gal-1 was more important than stromal Gal-1 to promote tumor growth, immunosuppression and metastasis [19]. Based on these findings, we hypothesize that Gal-1 expression in tumor cells may contribute to NSCLC progression and, although in our study the impact on survival was only of borderline significance when multivariate analysis was performed, we assume that the assessment of a larger cohort of patients will reveal a much more significant association. Furthermore, when higher expression in stroma and tumor cells was combined (high "total score"), the impact in survival reached statistical significance when multivariate analysis was used. Also, in a different multivariate analysis, in two separated tree-structured models, the "tumor cell percentage" and the "total score" discriminated survival to the best and were allocated to node I.

Besides global assessment of Gal-1 expression in the tumor stroma, we evaluated whether Gal-1 was particularly expressed in endothelial cells associated to tumor or normal tissue. We observed that about 50% of NSCLC samples showed Gal-1 expression in blood vessels within both tumor and normal tissue, showing an important correlation of Gal-1 expression in the two compartments. It is possible to speculate that factors secreted by these tumors may contribute to induce Gal-1 expression by endothelial cells in both tumor and normal tissue. This observation could be associated to the field of cancerization described in lung airways, where molecular abnormalities appear early in lung cancer pathogenesis

and are shared between the tumor and adjacent histologically normal tissue [32]. However, in spite of these possible outcomes, these parameters did not reach a significant impact on patients' survival.

In summary, in this study we have demonstrated using single or combined parameters of tumor evolution, the clinical relevance of Gal-1 expression in human NSCLC. Our results show that Gal-1 expression may be a useful biomarker for a better prediction of tumor evolution for optimizing adjuvant treatments. The development of novel molecular approaches targeting Gal-1 or its specific glycans will be of particular importance either alone or in combination with current therapeutic modalities in order to tailor more selective treatments for lung cancer patients.

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Conflict of interest statement

None declared.

Table 1 Clinicopathologic parameters in patients with NSCLC. The study included white individuals (median age, 64 years; range, 45-85; median survival at five years, 52.5 months; range, 1-60).

	Number of cases (%) [a]
Sex (n=103)	
Male	69 (66.9%)
Female	34 (33.0%)
Histologic type (n=96)	
Squamous Cell Carcinoma	24 (25.0%)
Adenocarcinoma	61 (63.5%)
Other	11 (11.5%)
Stage (n=101)	
Ia	29 (28.7%)
Ib	31 (30.7%)
IIa	24 (23.8%)
IIb	6 (5.9%)
IIIa	11 (10.9%)
Tumor size (n=103)	
1a	16 (15.5%)
1b	22 (21.4%)
2a	41 (39.8%)
2b	14 (13.6%)
3a	10 (9.7%)
Lymph node status (n=100)	
0	76 (76%)
1	16 (16%)
2	8 (8%)

[a]where columns do not sum to the total, data were missing or unknown

Table 2 Double entry table combining the percentage and intensity values of Gal-1 expression in NSCLC

Percentage value	Intensity value				
	0	**1**	**2**	**3**
0	0			
1		1	1	2
2		2	2	3
3		3	3	4

Table 3 Correlation between variables associated with Gal-1 expression and the main relevant parameters in lung cancer

		Tumor size	Lymph node status	Age
Tumor cell percentage	Correlation coefficient	0.039	-0.033	0.138
	p-value	0.695	0.745	0.166
Tumor stroma percentage	Correlation coefficient	-0.029	0.140	0.003
	p-value	0.770	0.164	0.974
Tumor cell score	Correlation coefficient	-0.007	-0.076	0.217
	p-value	0.948	0.452	0.028*
Tumor stroma score	Correlation coefficient	0.022	0.028	-0.033
	p-value	0.824	0.784	0.742
Tumor vessels	Correlation coefficient	-0.014	0.067	-0.067
	p-value	0.886	0.508	0.504
Normal vessels	Correlation coefficient	0.035	0.031	-0.047
	p-value	0.743	0.775	0.660
Total score	Correlation coefficient	0.110	-0.032	0.035
	p-value	0.270	0.755	0.725

Table 4 Cox proportional hazards model for the "total score" of Gal-1 expression in stage I-III NSCLC. Reference value: "total score"

	COEF β (95% CI)	P VALUE	RISK RATIO FOR MORTALITY
Lymph node status **(none/intrapulmonary)**	1.381 (1.801 – 8.782)	0.001	3.977
Histologic type **(ADC, SCC or other)**	0.758 (1.078 – 4.223)	0.030	2.133
Tumor size **(1a, 1b, 2a, 2b or 3a)**	0.763 (1.407 - 3.268)	0.000	2.144
Total score **(low/high)**	0.894 (1.011 - 5.914)	0.047	2.446

ADC: adenocarcinoma; SCC: squamous cell carcinoma

Legends to figures

Fig. 1 Representative microphotographs of Gal-1 expression in tumor cells (A,B); tumor stroma (C,D); and endothelial cells in the tumor (E,F) and normal (G,H) tissue in human non-small cell lung cancer (NSCLC). Inset shows Gal-1 expression in peripheral nerves used as an internal positive control (H). Arrows indicate endothelial cells delineating blood vessels. Scale bar: 50 μM

Fig. 2 Overall survival plots according to Kaplan-Meier's method for the groups defined on the basis of Gal-1 expression in tumor cells ("tumor cell percentage") (A; P value= 0.029) or expression in both tumor cells and stroma ("total score") (B; P value= 0.033)