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Nuclear galectin-3 expression is an independent predictive factor of recurrence for adenocarcinoma and squamous cell carcinoma of the lung

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The tumor stage is the most powerful prognostic tool for predicting the survival rates of lung carcinoma patients. However, prognosis of individual patients is difficult in part because of the marked clinical heterogeneity among such patients. Galectins are involved in cell growth, apoptosis and cell migration features, and their diagnostic and prognostic values have already been demonstrated in various types of cancers. In the present paper we analyze the potential prognostic value of immunohistochemical galectin-3 expression in lung adenocarcinomas and squamous cell carcinomas. In all, 165 squamous cell carcinomas and 121 adenocarcinomas were immunostained for galectin-3. In each case the immunohistochemical analyses consisted of an evaluation of the percentage of tumor cells stained and the intensity of staining. An IP score (ie Intensity × Percentage) was thus determined for each lung carcinoma. A large majority of cases displayed galectin-3 expression. While the cytoplasmic staining in the squamous cell carcinomas was focal and moderately intense, the staining in the adenocarcinomas was diffuse and intense. The IP scores were significantly ($P=0.0001$) higher in the adenocarcinomas than in the squamous cell carcinomas. The difference in nuclear expression profiles between the two cancer types was statistically significant ($P=0.0005$). Cox multivariate analysis carried out on the patients' genders, the TNM classification and the galectin-3-related variables showed that of the galectin-3-related variables, only the nuclear location of galectin-3 was identified as a prognostic indicator of recurrence independent of the clinicopathological features characterizing the patients ($P=0.02$). The prognostic contribution of this latter variable was enhanced when the patients with relapse-free follow-ups longer than 8 months were considered ($P=0.005$). Galectin-3 immunohistochemical expression differs between squamous cell carcinomas and adenocarcinomas, but the nuclear expression of galectin-3 behaves as a significant prognostic predictor for all the cases as a group.
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Lung cancer is a major cause of mortality and morbidity worldwide and thus constitutes a substantial public health problem in Western countries.[1,2] Determining the prognosis for an individual patient with a lung carcinoma is difficult, in part because of the marked clinical heterogeneity among the patients, even into a particular histological group. Currently, the most powerful prognostic tool for predicting the survival rates of these patients is their tumor stage.[3] Histopathological findings cannot adequately predict disease progression, especially for patients with stage I squamous cell carcinomas or adenocarcinomas who run the risk of developing metastasis despite surgical treatment and maximum therapy.[4] Modern literature accordingly emphasizes the search for new biological

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prognostic markers in order to refine the prognostic value contributed by tumor staging and histopathological analyses.[4–9]

Among the biological markers whose diagnostic and prognostic values have already been demonstrated in various types of cancers are galectins in general,[10–12] and galectin-3 in particular.[13,14] In all, 14 galectins have been identified to date[10,11] which function as cell receptors for the *N*-acetyl-lactosamine (LacNAC) moieties present on most of the extracellular matrix components.[15,16] Galectins are involved in cell growth, apoptosis and cell migration features.[17–20] Of these 14 galectins, galectin-3, a chimera-type galectin, is of special interest since its structure harbors a sugar-combining site, a collagen-sensitive stalk and a short *N*-terminated section, acting as a target for phosphorylation.[21] What is particularly intriguing about this sole chimera-type galectin is its presence in nuclear, cytoplasmic and extracellular sites,[22] its ability to interact with a variety of carbohydrate and protein ligands and to form pentamers with unique crosslinking abilities,[23] its strong antiapoptotic activity coupled with the nuclear export of the phosphorylated form (for example in chemotherapeutic treatment),[24] and its effect on promoter activity of cyclin D1,[25] which is a prognostic marker in the adenocarcinoma subgroup of lung carcinomas.[26]

Alteration of galectin-3 expression in lung carcinomas[27–29] and a high level of galectin-3 in the sera of lung carcinoma patients[30] have already been observed. However, a comprehensive analysis of the potential prognostic value of galectin-3 has not yet been carried out on a large series of lung carcinoma patients. We therefore analyzed the potential prognostic value of immunohistochemical galectin-3 staining, taking into account cytoplasmic *vs* nuclear galectin-3 location, on a retrospective series of 165 squamous cell carcinoma and 121 adenocarcinoma patients. We also analyzed the pattern of galectin-3 expression in the various histological structures of normal lungs.

Materials and methods

Patients and Tissue Samples

This study is a retrospective analysis of 286 patients who underwent the surgical resection of their squamous cell lung carcinomas ($n = 165$) or adenocarcinomas ($n = 121$) at the Erasmus University Hospital (Brussels, Belgium) between 1995 and 2001. The median patient follow-up period was 23 months (range = 0–86 months).

The clinical data available included age, gender, smoking habits, date of initial diagnosis, neoadjuvant therapy, histopathological diagnosis, pathological tumor stage, adjuvant therapy, recurrence and the date and etiology of death or the last clinical follow-up. These data are summarized in Table 1 and analyzed in the Results section. Hematoxylin- and eosin-stained histological sections were available for the 286 lung cancer cases and for 48 cases of lymph node metastases. The pathological features of each lesion were re-examined for a confirmation of the diagnosis and the selection of adequate specimens for immunohistochemical analysis. All the cases were classified according to the World Health Organization[31] histological criteria into: well, moderately or poorly differentiated squamous cell carcinomas; and acinar, papillary, solid, mixed or variant adenocarcinomas. The tumors were also classified according to the TNM classification (UICC 2002)[32] and staging was performed as follows: stage I (T1-2 N0 M0), stage II (T1-2 N1 M0 or T3 N0 M0), stage III (T1-2 N2-3 M0, T3 N1-3 M0 or T4 N0-3 M0) and stage IV (any T any N and M1).

Table 1 Clinicopathological data for 286 patients with primary squamous cell carcinomas or adenocarcinomas

Age (years), median (range)	65 (33–82)
Gender, female/male	64/222
Smoking habits[a]	
Smoker	246 (94%)
Nonsmoker	15 (6%)
Neoadjuvant therapy (23/286)	
Radiotherapy	1
Chemotherapy	19
Radiochemotherapy	3
Surgical treatment	
Segmentectomy	17 (6%)
Lobectomy	208 (73%)
Pneumonectomy	61 (21%)
Histology	
Squamous cell carcinoma	165 (58%)
Well differentiated	17 (10%)
Moderately differentiated	87 (53%)
Poorly differentiated	61 (37%)
Adenocarcinoma	121 (42%)
Acinar	70 (58%)
Papillary	5 (4%)
Solid	31 (26%)
Mixed	12 (10%)
Variants	3 (2%)

[a]Smoking habits were unknown for 25 cases.

Immunohistochemistry

Five-μm-thick formalin-fixed and paraffin-embedded tissue sample sections were deparaffinized in xylene and treated with 0.3% hydrogen peroxide in methanol for 30 min in order to block endogenous peroxidase activity. A 1/100 dilution of a rabbit polyclonal antibody specific to human galectin-3 was then applied as a primary antibody at 4°C overnight. This was followed by a standard staining procedure using the 'Immunologic Ultrasense-kit' (Immunologic, Duiven, NL). The polyclonal antibody against galectin-3 produced by recombinant expression with the expression vector prCBP35, kindly provided by Dr JL Wang (East Lansing, MI,

USA) was raised as described previously.[18] The specificity of the galectin-3 antibody was checked by immunoblotting and ELISA assays with controls to exclude any crossreactivity to other galectins (especially galectin-1, which is abundant in human lungs), as previously detailed.[18–20,33] An internal positive control consisted in the expression of galectin-3 (also labeled Mac-2) by alveolar macrophages. The negative control sections were immunostained under the same conditions, but with the omission of the primary antibody. The sections were counterstained with haematoxylin.

Evaluation of Immunohistochemical Staining

The sections were assessed by means of standard light microscopy by two independent observers (AM, MR). When discrepancies were encountered, the cases were settled by consensus with a third observer (IS). To each case the immunohistochemical analyses consisted of an evaluation of (i) the percentage of tumor cells stained (no staining = 0; $<60\% = 1$; $>60\% = 2$), (ii) the pattern of expression (no staining = 0; focal staining = 1; diffuse staining = 2) and (iii) the intensity of staining (no staining = 0; weak to moderate staining = 1; strong staining = 2). The location of galectin-3 in cells (nuclear and/or cytoplasmic staining) and the presence of stromal staining were also ascertained. The IP score (ie Intensity $\times$ Percentages) was determined as detailed by van den Brule *et al*,[34] ie by multiplying the percentage of positively stained tumoral cells by the strength of the staining. This score was used to determine the overall galectin-3 expression patterns in the primary tumors and the lymph node metastases. The values provided by the IP score therefore include 0 (absence of any staining), 1 (fewer than 60% of weakly to moderately stained cells), 2 (either less than 60% of strongly stained cells or more than 60% of weakly to moderately stained cells) and 4 (more than 60% of strongly stained cells). The IP value cannot be 3 because it is impossible to obtain this value by multiplying the percentage score by the staining score.

Statistical Analysis

Independent groups of numerical data were compared by means of the nonparametric Kruskal–Wallis (more than two groups) or Mann–Whitney (two groups) tests. The relationships between the qualitative (or ordinal) variables analyzed were studied by means of contingency tables. The significance of the potential associations was evaluated by means of χ^2 tests (after grouping the feature scores which concerned too few cases in order to ensure the statistical validity of the test) or the exact Fisher's tests (in 2×2 cases only). The nonparametric Spearman's correlation analysis was used to establish correlations between the ordinal variables.

Standard relapse-free and overall survival analyses were carried out by means of Kaplan–Meier curves and Gehan's generalized Wilcoxon test. Standard Cox regression analyses were also used to fit an explanatory model (generated on the basis of different variables analyzed in the study) to the relapse-free and overall survival data. This enabled the possible simultaneous influence of several variables on the survival period to be tested. All the statistical analyses were carried out using Statistica (Statsoft, Tulsa, OK, USA).

Results

Clinicopathological Features

The clinicopathological data of the 286 patients are summarized in Table 1. The women, who constituted 23% of the patients, were significantly younger than the men ($P = 0.02$). Of the 261 patients, on whose smoking habits data were available, a significant difference between males and females was observed: 19% of the women against 2% of the men were nonsmokers ($P = 0.00002$). Adenocarcinomas constituted the most frequent type of tumor in the women ($n = 40/64$, ie 63%; $P = 0.0003$ when compared to the men, for whom $n = 81/222$, ie 36%).

Multivariate Cox regression analysis was used to identify the independent clinicopathological factors, which might have a significant influence on relapse-free and overall survival periods. The results are shown in Table 2. The age, the T (coded as 1 = T1, 2 = T2 and 3 = T3 or T4) and the N (coded as 0 = N0, 1 = N1, 2 = N2 and 3 = N3) variables were considered as being numerical. Gender and histological tumor type were used in a binary format as follows: male *vs* female and adenocarcinomas *vs* squamous cell lung carcinomas. Table 2 indicates that sex and T stage were independent prognostic factors of recurrence (see P^a-values in Table 2). These results were supported by the Kaplan–Meier analyses (see P^b-values in Table 2). These analyses show that factors such as being female and having a high T stage (to a lesser extent) are significantly associated with a decrease in the relapse-free survival period (data not shown). The overall survival periods analyzed by the Cox regression analysis indicated that the factors contributing to significant independent prognostic information are age and T status (see associated P^a-values in Table 2). These results were supported by the Kaplan–Meier studies (see P^b-values in Table 2, except for age which is a continuous variable), which also revealed that an increased N status was associated with a poor prognosis ($P = 0.02$) and the adenocarcinomas were associated with a slightly more favorable outcome ($P = 0.04$) than the squamous cell carcinomas (data not shown). However, these two latter factors did not contribute any independent prognostic information (see Cox analysis).

Expression of Galectin-3 in Normal Lung

Bronchial epithelial cells expressed galectin-3 strongly and the chondrocytes of the bronchial cartilage displayed strong nuclear staining. Whereas the pneumocytes of the alveolar wall showed light/moderate staining, the alveolar macrophages were strongly stained. The interstitial fibroblasts had a staining level comparable to the pneumocytes (data not shown).

Differential Expression of Galectin-3 in Adenocarcinomas and Squamous Cell Lung Carcinomas

The results of the immunohistological assessments of all the tumors are detailed in Table 3 and illustrated in Figure 1. The majority of cases (260/286 = 91%) exhibited immunohistochemical galectin-3 expression. Only 12 (7%) squamous cell lung carcinomas and 14 (12%) adenocarcinomas did not display any such expression. As detailed below, the patterns of galectin-3 expression were different between the two carcinoma groups. This was statistically confirmed by the *P*-values detailed in Table 3.

While the cytoplasmic staining was focal and moderately intense in the squamous cell lung carcinomas (Figure 1a), the staining in the adenocarcinomas was rather diffuse and intense (Figure 1c–d). This is why the IP scores (Figure 2) were greater in the case of the adenocarcinomas ($P = 0.0001$). We observed no difference between the different adenocarcinoma subtypes (data not shown). In the case of the squamous cell lung carcinomas, the staining was comparatively intense in well-differentiated areas (Figure 1b). In a limited number of squamous cell lung carcinomas (41/165 = 25%) a nuclear expression of galectin-3 was observed and, when such nuclear expression was present, it concerned a small number of nuclei. In contrast, nuclear staining was present in 45% (54/121) of the adenocarcinomas (without having any difference between the different subtypes; Figure 1c–d). This difference in the profiles of nuclear expression between the two carcinoma groups was detected as being very significant ($P = 0.0006$). In contrast, the stromal staining did not differ across the two groups (Table 3). The expression of galectin-3 expression in the tumors from the patients treated with a neoadjuvant therapy was not different when compared with the situation of those patients who had not undergone any preoperative treatment (data not shown). The IP score was similar for the primary tumors and their lymph node metastases in 54% (26/48) of the cases. When a difference existed, the IP score tended to be slightly higher in the case of the metastases as opposed to the primary tumors ($P = 0.09$).

Table 2 Multivariate Cox and Kaplan–Meier analysis

Features	*Relapse-free survival*		*Overall survival*	
	$P^{a\ (Cox)}$	$P^{b\ (Kaplan–Meier)}$	$P^{a\ (Cox)}$	$P^{b\ (Kaplan–Meier)}$
Age	NS	—	**0.01**	—
Gender	**0.003**	**0.001**	NS	NS
Histology	NS	NS	NS	**0.04**
T	**0.02**	**0.01**	**0.03**	**0.02**
N	NS	NS	NS	**0.02**

Features analyzed by means of standard Cox regression analyses to fit an explanatory model to the relapse-free survival data on the one hand and the overall survival data on the other, in order to test the possible simultaneous influences of all these features on (relapse-free or overall) survival periods. The P^a-values are the significance levels associated by these multivariate analyses with the features: $P^a < 0.05$ (in bold characters) means that the feature is associated with significant prognostic values independently of the other features taken into account.
The prognostic values (with respect to relapse-free and overall survival) of each feature have also been analyzed by means of standard Kaplan–Meier analyses (except 'Age' which is a continuous feature) using Gehan's generalized Wilcoxon test (two groups) or the standard Mantel procedure (more than 2 groups). The P^b-values are the significance levels established by these latter univariate analyses (which took only one feature into account on each occasion): $P^b < 0.05$ (in bold characters) means that the survival curves characterizing the different patient groups determined by the feature analyzed are significantly different.
NS = not significant, that is, *P*-values > 0.05.

Prognostic Value of Galectin-3 Expression

Multivariate Cox statistical analysis carried out on the patients' genders, the TNM classification and the galectin-3-related variables showed that of the galectin-3-related variables, only the nuclear

Table 3 Galectin-3 expression in lung carcinomas

	Squamous cell carcinoma	*Adenocarcinoma*	P
Percentage of cells (0%, <60%, >60%)	7/46/47	12/28/60	**0.01**
Pattern staining (no, focal, diffuse)	7/40/53	12/23/65	**0.01**
Intensity (no, moderate, strong)	7/85/8	12/68/20	**0.003**
Cytoplasmic/nuclear staining	75/25	55/45	**0.0006**
Stromal staining (absence, presence)	62/38	69/31	0.18

Distribution of the frequencies (%) observed in our series for each value of the five variables analyzed (detailed under Materials and methods), together with their associated *P*-values (χ^2 test or exact Fisher's test in 2×2 cases).
The significant *P*-values are shown in bold characters.

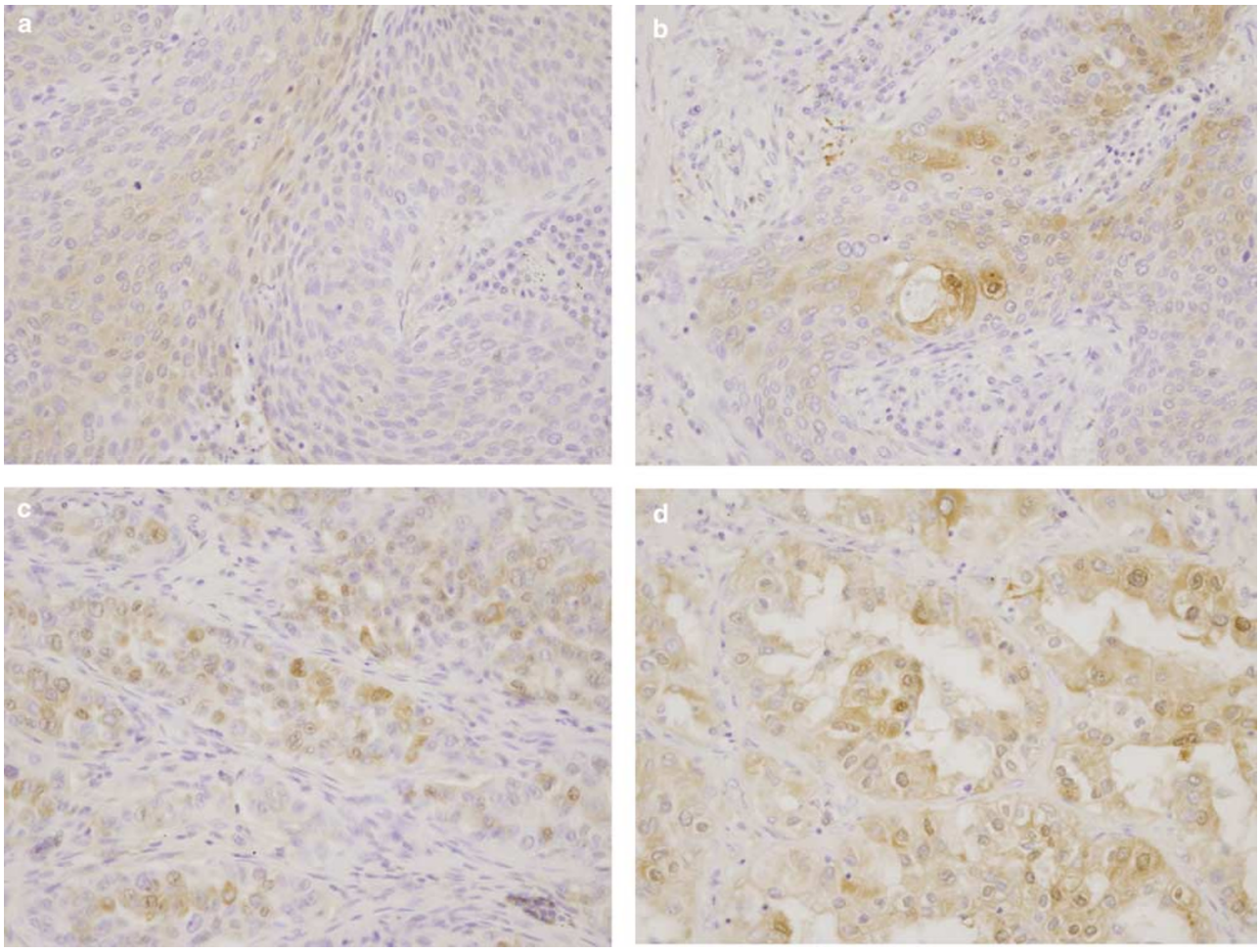

Figure 1 Pattern of galectin-3 expression in squamous cell carcinomas and adenocarcinomas. (**a**) Illustration of the weak to moderate staining in the cytoplasm of a squamous cell carcinoma. (**b**) An increased expression is observed in the well-differentiated areas, particularly in the keratin pearls of a squamous cell carcinoma. A solid (**c**) and an acinar (**d**) adenocarcinoma, both of which show diffuse and strong cytoplasmic and nuclear staining.

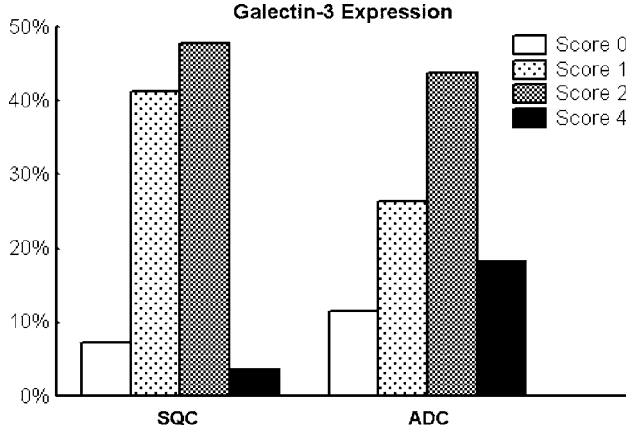

Figure 2 Pattern of galectin-3 expression illustrated by the IP scores which reveal differences across the two different histological types (χ^2 test: $P = 0.0001$). SQC = squamous cell carcinoma; ADC = adenocarcinoma.

location of galectin-3 was identified as a prognostic indicator of recurrence independent of the clinicopathological features characterizing the lung carcinoma patients ($P = 0.02$). As illustrated in Figure 3, Kaplan–Meier and Cox analyses evidenced that the best prognostic value associated with this galectin-3 feature concerned the patients with relapse-free follow-ups longer than 8 months ($n = 180$; $P = 0.005$).

Discussion

Both squamous cell carcinomas and adenocarcinomas are conceptualized as groups of heterogeneous clinical entities that share similar molecular and cellular origins but have different clinical behavior patterns and hence different prognoses. Brundage *et al*[3] have scrutinized the literature of the past decade on prognostic factors in the case of nonsmall cell lung cancer patients. They conclude that while the breadth of the prognostic factors studied in the literature is extensive, the scope of the factors evaluated in individual studies is narrow. More extensive studies with clinically relevant models are required to prove the merit of the proposed

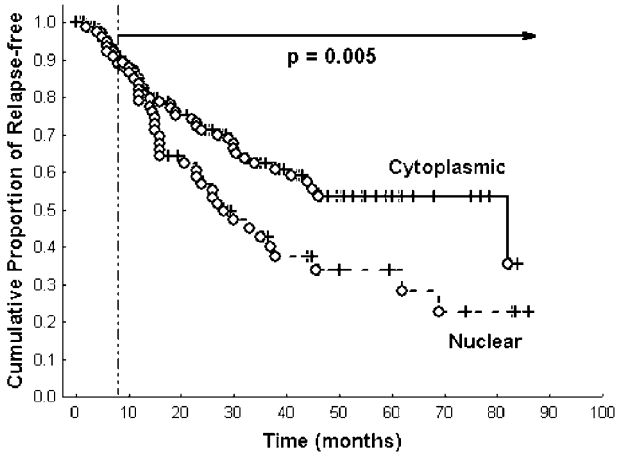

Figure 3 Relationship between the nuclear expression of galectin-3 and the relapse-free survival periods. The relapse-free survival curves are displayed according to the presence/absence of galectin-3 in the tumor cell nuclei. The dots symbolize the recurrences and the crosses the relapse-free cases, respectively. The vertical line and the arrow indicate the patients' subpopulation on which the *P*-value was computed (by means of the Gehan's generalized Wilcoxon test), that is, the patients with relapse-free follow-ups of at least 8 months.

prognostic factors in establishing the management of patients with lung carcinomas. Some recent studies are based on an analysis of large series of cases of this type. For example, Pujol *et al*[9] performed a meta-analysis based on individual updated data from published or unpublished controlled studies dealing with the prognostic significance of the serum CYFRA 21-1 (a cytokeratin 19 fragment) level at any tumor stage (nine institutions, 2063 patients). A high pretreatment CYFRA 21-1 level emerged as an unfavorable prognostic determinant at the 1-year end point, and, remained an unfavorable prognostic determinant at the 2-year end point (together with a locally advanced stage).[9] Au *et al*[26] submitted a panel of 18 immunomarkers to unsupervised hierarchical clustering analysis to investigate the possibility of identifying different subgroups in a series of 284 patients with nonsmall cell lung cancers. These authors report that the analysis of the three different World Health Organization (WHO) subtypes (namely, adenocarcinomas, squamous cell carcinomas and large cell carcinomas) showed that different markers were significant in different subtypes, but did not behave as significant prognostic predictors in the case of these carcinomas as a group.[26] In the present study, we observed that the nuclear location of galectin-3 behaves as a significant prognostic predictor relative to adenocarcinomas and squamous cell carcinomas as a group.

In normal lungs galectin-3 expression in the epithelial lining increases during the fetal development of the bovine respiratory tract, a modification which is associated with a shift from the primary nuclear location to the cytoplasm.[35] A similar epithelial phenotype (increased galectin-3 expression in pneumocytes) occurs during irradiation-induced lung injury.[36] Nonsmall cell lung cancers were seen to harbor increased levels of galectin-3 mRNA when compared to normal epithelial cells.[27] In the present study, we observed different patterns of galectin-3 expression between squamous cell carcinomas and adenocarcinomas. Squamous cell carcinomas express galectin-3 predominantly in the cytoplasm of well-differentiated areas with rare nuclear staining. On the contrary, about 50% of adenocarcinomas express galectin-3 in both the nucleus and the cytoplasm, with more marked staining than in the case of squamous cell carcinomas. Galectin-3 expression is not significantly different in lymph node metastases and primary tumors and is not modified by neoadjuvant therapy. We also observed that the nuclear location of galectin-3 expression is an independent predictor of recurrence in nonsmall cell lung cancer, in particular in the case of patients having relapse-free follow-ups longer than 8 months. To our knowledge this is the first study to have evidenced a correlation between galectin-3 expression and prognosis in lung carcinomas. In fact, the subcellular location of galectin-3 correlates with its phosphorylation status. Two isoelectric variants of galectin-3 have been described, namely: a phosphorylated and a nonphosphorylated form, and evidenced phosphorylation as a requirement for its nuclear export.[24] While an association between the cellular location of galectin-3 expression and the stage of tumoral progression has been reported for cancers of the endometrium, the prostate and the colon,[37] enhanced cytoplasmic expression of galectin-3 in tongue carcinomas was established as a predictor factor of disease recurrence.[38] In nuclei, galectin-3 (with galectin-1) constitutes part of an interacting dynamic network of factors involved in the splicing and transport of mRNA.[16,22] The nuclear *vs* cytoplasmic distribution of the protein is dependent on the proliferation state of the cells: whereas galectin-3 is predominantly cytoplasmic in quiescent cultures of 3T3 fibroblasts, proliferating cultures of the same cells show intense nuclear staining.[39] In nuclei, galectin-3 is detected in interchromatin granule clusters that have been suggested as being storage sites for splicing factors.[39] Such speckles have been observed for a number of nuclear antigens including the Sm polypeptides of small nuclear ribonucleoprotein particles that play a role in the splicing of pre-mRNA. The nuclear location of galectin-3 is also associated with transcriptional regulation in the case of transformed thyroid cells.[40]

In conclusion, the present study shows that whereas galectin-3 expression differs between squamous cell carcinomas and adenocarcinomas, the nuclear expression of galectin-3 behaves as a significant prognostic predictor for all the cases as a group.

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