The determination of the levels of circulating galectin-1 and -3 in HNSCC patients could be used to monitor tumor progression and/or responses to therapy

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Summary To evaluate galectin-1, -3 and -7 serum levels as diagnostic and/or prognostic markers for head and neck squamous cell carcinomas (HNSCCs). ELISA was employed to test sera from 102 patients with HNSCCs and from 38 healthy control volunteers for galectin-1, -3 and -7 serum levels. Serum galectin levels were assayed by ELISA and the levels of galectin expression in HNSCCs were determined by means of immunohistochemistry. HNSCCs display significant immunohistochemical amounts of galectin-7, but this galectin cannot be detected in the blood of HNSCC patients. Galectin-3 levels differ significantly (p = 0.03) in healthy volunteers and HNSCC patients. Using a threshold value of 4.3 ng/ml, galectin-3 serum level enabled a significant level of discrimination (p = 0.03) to be established between the cancer patients and the healthy volunteers, with 90% level of specificity and 36% level of sensitivity. The discrimination was even better when using a threshold value of 13.5 ng/ml for galectin-1 (p = 0.001), with 100% level of specificity and 22% level of sensitivity. A subgroup of stage IV HNSCC patients

KEYWORDS Galectins-1, -3, -7; Head cancer; Neck cancer; Serum; ELISA

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Introduction

Head and neck squamous cell carcinomas (HNSCCs) are the sixth most common form of cancer worldwide and constitute the most common malignant neoplasm arising in the mucosa of the upper aero-digestive tract.1,2 The survival rates have not shown any improvement over the past 30 years.1,2 Fifty percent of HNSCC patients succumb to their disease, while each year 5% develop additional primary tumors.2 Furthermore, HNSCCs have a severe impact on patients’ quality of life.3,4 As emphasized by Hunter et al.,1 the significant morbidity subsequent to treatment often calls for long-term multidisciplinary care, and this leads to a significant financial burden for the treating institutions. Nearly two-thirds of HNSCC patients have advanced stages (III and IV) of the disease and, despite the use of resection and postoperative concomitant chemotherapy and radiotherapy, high-risk HNSCCs frequently recur in original tumor beds or secondary metastasis.1–4 Novel approaches are thus necessary to provide head and neck oncologists with a more effective armamentarium against this challenging disease. In this context, the application of proteomics and bioinformatic analysis has facilitated the identification of critical phenotypic changes in HNSCCs.2

Galec
tins could be of potential interest in identifying subgroups of HNSCC patients with distinct prognoses. Galec
tins are animal lectins defined by shared-consensus amino acid sequences and a high binding affinity for β-galactose-containing oligosaccharide chains.5,6 To date, 15 mammalian galec
tins have been identified and numbered consecutively in order of discovery (galec
tin-1 to galec
tin-15).6 Galecticins play a number of important roles in cancer biology because they contribute to neoplastic transformation, tumor cell survival, angiogenesis and tumor metastasis.5,6 We recently reviewed the roles of galec
tins as modulators of HNSCC progresssion.7 Three galec
tins, i.e., galec
tin-1, -3 and -7, seem to emerge as major players in HNSCC biology.7 Recent reviews focus specifically on the biological roles played by galec
tin-1,8,9,10 and -711 in general, and not only in the context of HNSCCs. With respect to HNSCCs, He et al.12 observed an increase in galec
tin-1 expression in tongue SCCs as compared to their matched normal mucosa. The level of expression of galec
tin-1 contributes to the prognosis of the recurrence of laryngeal tumors and, to a lesser extent, of pharyngeal tumors after surgery, and to patients’ survival prospects.13,14 We were the first to show that a decrease in the extent of the expression of galec
tin-3 and galec
tin-3 binding sites correlates significantly with an increasing level of clinically detectable HNSCC aggressiveness,15 a finding that has been validated further by other groups for tongue cancers,16 oropharyngeal cancers17 and laryngeal cancers.18 In high stage hypopharyngeal squamous cell carcinomas, galec
tin-3 expression is not related to prognosis.14 Galecticin-7 is overexpressed in buccal squamous cell carcinomas as compared to normal buccal epithelia.19

The aim of the present study is to investigate whether the levels of circulating galec
tin-1, -3 and -7 are modified during HNSCC progression or only in subgroups of patients undergoing specific therapeutic regimens. For this purpose, we determined such circulating galec
tin concentrations by means of ELISA on a series of 102 HNSCC patients and 38 healthy volunteers. The present study must thus be considered as a preliminary report demonstrating the feasibility of quantifying galec
tins in the blood of HNSCC patients, with the ultimate aim of using these dosages on specific subset of patients to monitor their disease.

Materials and methods

Sera and patients

Blood samples were obtained from patients admitted to the Department of Otalaryngology and Head and Neck Surgery of the Centre Hospitalier Universitaire Saint-Pierre (Brussels). The patients and the healthy control subjects were asked to participate in the study as approved by the Saint-Pierre Institutional Review Board. Informed consent and a medical history were obtained from each patient. A total of 102 patients with histologically proven diagnoses of cancer located in the head and neck area were enrolled in this study. The sera were stored at −20°C until assay. Galec
tin-1 and -3 serum levels were measured before and after the treatment of the primary tumors or the recurrences. Moreover, the sera were tested every 2 months post-treatment over a maximum follow-up period of 24 months. Samples were also obtained from 38 matched healthy blood donors with a mean age of 51 years (range, 30–90 years).

Enzyme-linked immunosorbent assay for galec
tin-3 (ELISA)

Ninety-six-well microplates were pre-coated with capture polyclonal antibody (goat) to human galec
tin-3 (Human galec
tin-3 ELISA, BenderMedSystems, BMS279, Vienna, Austria) and washed three times with wash buffer [1% Tween 20 (Sigma–Aldrich, St. Louis, MO) in PBS]. Samples (50 µl) were...
added in duplicate to each well, which contained 50 µl of sample diluent. Detection antibody diluted in Reagent Diluent was then added to each well and incubated at room temperature (RT) for 2 h on a microplate shaker set at 200 rpm. The wells were then washed three times before adding 100 µl of horseradish peroxidase-conjugated anti-rabbit IgG. After 1 h of incubation at RT, wells were washed three times and 100 µl of tetra-methylbenzidine substrate solution were added. After a 20-min incubation period at RT the reaction was stopped by means of 100 µl of stop solution. The absorbance of each sample was determined at 450 nm in a Labsystems Multiskan MS microplate reader (Thermo Electron Co, Zellik, Belgium). A standard curve ranging from 0.156 to 10 ng/ml of galectin-3 (BMS279, Vienna, Austria) was generated for each ELISA.

Galectins-1 and -7 ELISA

Ninety-six-well microplates were coated with capture antibody at 2000 ng/ml (100 µl/well, galectin-1: AF1152; galectin-7: AF1339, R&D Systems, Minneapolis, MN). After incubation overnight at RT, the wells were washed three times with wash buffer [0.05% Tween 20 (Sigma–Aldrich, St. Louis, MO) in PBS]. Samples (100 µl) were added to the wells and the mixture incubated at RT for 2 h and aspirated. The wells were then washed with wash buffer. Detection antibody (100 ng/ml, 100 µl; galectin-1: BAF 1152, galectin-7: BAF 1339, R&D Systems) diluted in Reagent Diluent (R&D Systems, Minneapolis, MN; PBS, 5% Tween 20, 2% goat serum) was added to each well and the microplate incubated at RT for 2 h. The wells were then washed three times and 100 µl of streptavidin–horseradish peroxidase (1:200) added. After 20 min of incubation at RT the plate was developed and the absorbance of each sample was determined at 450 nm. A standard curve ranging from 5 to 160 ng/ml of galectin-1 and from 1.25 to 40 ng/ml of galectin-7 (R&D Systems) was generated for each ELISA.

Immunohistochemical procedure

As detailed elsewhere, tissue sections were immunostained following a slightly modified version of the streptavidin–biotin immunoperoxidase method (ABC method). Briefly, antigen retrieval was achieved by microwave treatment of dewaxed sections in 0.01 M citrate buffer (pH 6.0) for 2 × 5 min at a power setting of 900 W. After the microwave treatment the sections were incubated in 0.4% hydrogen peroxide for 5 min to block endogenous peroxidase activity, and thoroughly rinsed in PBS (0.04 M Na₂HPO₄, 0.01 M KH₂PO₄ and 0.12 M NaCl, pH 7.4). The sections were then successively exposed to avidin (0.1 mg/ml in PBS) and biotin (0.1 mg/ml in PBS) for 20 min to block the reactivity of endogenous biotin. After rinsing in PBS, they were incubated in 0.5% casein in PBS (CAS–PBS) for 20 min and exposed sequentially at room temperature to the following reagents solutions: (1) a primary antibody for 1 h; (2) biotinylated goat anti-rabbit IgG (diluted 1:50) for 30 min and (3) ABC complexes for 30 min. Specifically bound peroxidase activity was visualized by incubation with 0.02% 3,3’-diaminobenzidine–0.01% H₂O₂ in PBS. After each step of the immunostaining procedure the sections were rinsed in PBS to completely remove excess reagents. The sections were finally counterstained with PAS, hemalun and luxol fast blue and mounted in a permanent medium. Controls for the specificity of the immunolabeling included either the omission of the primary antibody or the substitution of non-immune sera in place of the primary antibodies to assess antigen-independent staining. In each case these controls were negative.

Data analysis

Data obtained from independent groups were compared by non-parametric Kruskal–Wallis (more than two groups) or Mann–Whitney U tests (two groups). The standard survival time analyses were performed using Kaplan–Meier curves. Standard Cox regression analysis was also used to fit to the survival data the explanatory models generated on the basis of the variables analyzed in this study. This protocol enabled us to test any possible simultaneous influence of several variables in relation to the survival period. The statistical analysis was performed by using the software Statistica (Statsoft, Tulsa, USA).

Results

Validation of the ELISA assays

Signal/concentration curves obtained with the galectin-1, -3 and -7 peptides were linear in a range of 5–160 ng/ml for galectin-1 (Fig. 1A), from 0.156 to 10 ng/ml for galectin-3 (Fig. 1B) and from 1.25 to 40 ng/ml for galectin-7 (Fig. 1C). No cross-reactivity was observed with the galectin ELISA assays based on galectin peptides (data not shown). For examples, no cross-reactivity was observed with the galectin-1 ELISA assay based on galectin-3 and -7 peptides.

HNSCCs display significant immunohistochemical amounts of galectin-7, but this galectin cannot be detected in the blood of HNSCC patients

While we succeeded in establishing a validated ELISA signal/concentration curve for galectin-7 (Fig. 1C), we failed to demonstrate the presence of galectin-7 in the serum from both the healthy individuals and the HNSCC patients (data not shown). However, as reported previously, HNSCCs display large amounts of galectin-7, a feature which is also illustrated here. Indeed, Figure 2A illustrates the pattern of Galectin-1 expression in the HNSCC of a patient in whose blood we detected large amounts of galectin-1, i.e., 30 ng/ml. The peritumoral area of this HNSCC also showed strong galectin-1 immunostaining (Fig. 2B). The immunohistochemical amounts of galectin-3 (Fig. 2C) and galectin-7 (Fig. 2D) were as high as those for galectin-1 (Fig. 2A) in this HNSCC. However, while we detected significant amounts of galectin-1 (see above) and galectin-3 in the blood of this patient (5.6 ng/ml), we failed to evidence any amounts of galectin-7 in his blood.
The level of galectin-1 in the sera of the healthy individuals varied between 0.7 and 13.4 ng/ml (median, 5 ng/ml) while the level of galectin-1 in the sera of the cancer patients varied between 0.1 and 30.8 ng/ml (median, 6.2 ng/ml). The galectin-1 serum levels were not significantly different in the cancer patients as compared to the healthy individuals. Yet, with a threshold value of 13.5 ng/ml, the galectin-1 serum level allowed one to significantly discriminate a subpopulation of cancer patients \((p = 0.001)\) with a specificity of 100% and a sensitivity of 22% (Fig. 3).

Figure 1 For each ELISA a standard curve was generated that ranged from 5 to 160 ng/ml of galectin-1 (A), from 1.25 to 40 ng/ml for galectin-7 (C), and from 0.156 to 10 ng/ml for galectin-3 (B) (variable on the y-axis), the absorbance of each sample was determined at 450 nm (variable on the x-axis).

Galectin-1 serum level in the normal subjects and in the HNSCC patients

The clinical data for the group of HNSCC patients for whom we evidenced high serum levels of galectin-1 are summarized in Table 2. Thirteen patients of this group of 15 HNSCC patients included stage IV HNSCCs. Two patients had peculiar clinical histories: the first (number 4 in Table 2) presented two synchronous primaries and the second (number 5) had a second primary cancer of the lung. As illustrated in Figure 4, serum galectin-1 was measured before and after treatment in eleven cases in this group of 15 patients. In all 11 patients there was a significant \((p = 0.003)\) decrease in the galectin-1 serum concentrations post-treatment as compared to the galectin-1 concentrations pre-treatment.

Galectin-3 serum level in the normal subjects and in the HNSCC patients

The level of galectin-3 in the sera of the healthy individuals varied between 1.3 and 6.4 ng/ml (median, 2.39 ng/ml) while the levels of galectin-3 in the sera of the cancer patients varied between 0.8 and 7.9 ng/ml (median, 3.2 ng/ml). The mean galectin-3 serum level in the cancer patients was significantly higher than in the case of the healthy individuals \((p = 0.03)\). With a threshold value of 4.3 ng/ml, the serum level allowed one to significantly \((p = 0.03)\) discriminate a group of HNSCC patients with a 90% level of specificity and a 36% level of sensitivity (Fig. 3). No statistically significant correlations were evidenced between the clinical features detailed in Table 1 (i.e., the tumor sites, the differentiation status and TN status) and the galectin-3 blood levels (data not shown).

Galectin-3 serum level differs in localized as opposed to metastasizing HNSCCs

The mean galectin-3 concentration values determined in the sera from the five HNSCC patients with metastatic diseases were significantly higher \((p = 0.01)\) than the mean concentration values determined in the sera from the 97 HNSCC patients with localized tumors.

Contribution of galectins 1 and 3 to the prognosis of survival consecutive to HNSCCs

We compared the survival curves of a group of patients with high galectin-1 (>13.5 ng/ml) or high galectin-3 (>4.3 ng/ml) serum levels with a group of patients with low galectin-1 (<13.5 ng/ml) and low galectin-3 (<4.3 ng/ml) serum levels. Our analysis showed that the high galectin-1 and -3 serum levels were associated with a weak, but nevertheless significant, prognostic value in terms of periods of survival for HNSCC patients (Fig. 5).

Discussion

As stated in a recent review, determining the levels of immunohistochemical expression of various types of galectins, including Galectin-1, -3, and -7, is of prognostic value for various subsets of HNSCC patients. Monitoring the levels of circulating galectin-1 and -3 could be of added value at clinical level if these concentrations are modified during the progres-
sion of the disease or in relation to its treatment. Iurisci et al.\textsuperscript{20} undertook the proof of concept in developing an immunoligand assay for the determination of the circulating levels of galectin-3 in cancer patients as compared to healthy controls. These authors observed that galectin-3 serum levels in patients with breast, gastrointestinal, lung or ovarian cancers, melanomas, and non-Hodgkin’s lymphomas are significantly high.\textsuperscript{15} They also demonstrated that galectin-3 serum concentrations are higher in sera from metastatic patients than in sera from patients with localized tumors.\textsuperscript{15} We observed the same feature in the present study with respect to the HNSCCs. Our study concentrated on galectin-1, -3 and -7 for the following reasons.

Le et al.\textsuperscript{13} recently demonstrated that galectin-1 is a hypoxia-regulated protein in HNSCCs. The microenvironment of solid tumors, including HNSCCs, possesses hypoxic regions that are not found in normal tissue.\textsuperscript{13} Hypoxia causes cellular resistance to conventional chemotherapy and accelerates malignant progression in cancer in general and in HNSCCs in particular.\textsuperscript{13,21,22} The expression of endogenous hypoxia markers in the case of the HIF-1 and HIF-2 pathways in HNSCC patients is strongly associated with the failure of radiotherapy.\textsuperscript{22} Using immunohistochemical methods it is possible to identify subgroups of HNSCC patients who are highly curable with radiotherapy or who are excellent candidates for clinical trials on hypoxia-targeting drugs in two different pathways.\textsuperscript{22} The study by Le et al.\textsuperscript{13} presents a new mechanism that shows how hypoxia can affect the malignant progression and therapeutic response of solid tumors such as HNSCCs by regulating the secretion of proteins, including galectin-1 which, in their turn, modulate immune

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**Figure 2** Immunohistochemical staining specific to galectins-1 (A, B), -3 (C) and -7 (D) in sections of stage IV (A–D) laryngeal squamous cell carcinomas. The peritumoral cells are also positive for galectin-1 (black arrows) (B). Magnification $\times$320.

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**Figure 3** Quantitative determination of the galectin-3 serum concentrations (variable on the x-axis) and the galectin-1 serum concentrations (variable on the y-axis) detected in a series of 102 HNC patients and 38 healthy individuals. With a threshold value of 13.5 ng/ml, galectin-1 serum level allowed one to significantly discriminate cancer patients ($p = 0.001$) with a 100% level of specificity and a 22% level of sensitivity. With a threshold value of 4.3 ng/ml, galectin-3 serum level allowed one to significantly discriminate cancer patients ($p = 0.03$) with a 90% level of specificity and a 36% level of sensitivity. Moreover, the galectin-1 and -3 concentrations in the sera from the patients with metastatic diseases (black arrows) were higher than in the sera from the patients with localized tumors.
privilege. Indeed, galectin-1 has been shown to inhibit the T-cell effector function by promoting T-cell apoptosis, by blocking T-cell activation and by inhibiting the secretion of proinflammatory cytokines. Galectin-1 confers immune privilege on experimental melanomas by the modulation of T-cell survival. The targeted in vivo inhibition of galectin-1 expression renders mice resistant to tumor challenge, a process requiring an intact CD4+ and CD8+ T-cell response. The cytoidal effects of galectin-1 on immune cells probably results from the binding of galectin-1 to T-cell-surface glycoproteins including CD2, CD3, CD7, CD43 and CD45. The signal transduction events that lead to cell death induced by galectin-1 in activated T-cells involve a number of intracellular mediators. The development of HNSCCs is strongly influenced by the host immune system. Recent evidence suggests that the anti-tumor responses of the HNSCC patients were compromised in the presence of functional defects or the apoptosis of T cells, both circulating and tumor-infiltrating. Immune cell dysfunction in HNSCC patients appears to extend far beyond the tumor microenvironment because both functional defects and massive lymphocyte death have also been observed in the peripheral circulation of patients with advanced HNSCCs.

Honjo et al. analyzed galectin-3 expression in 77 tongue specimens (including 54 squamous cell carcinomas and 23 specimens of normal mucosa) and observed that their levels of nuclear expression of galectin-3 decreased noticeably during the progression from normal to cancerous states while the cytoplasmic expression increased. The enhanced expression of galectin-3 in the cytoplasm was associated with a reduced level of disease-free survival in the case of these tongue cancer patients. Piantelli et al. evaluated the prognostic value of galectin-3 expression in 73 node-negative laryngeal squamous-cell carcinomas and observed

<table>
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<tr>
<th>Table 1</th>
<th>Patient population characteristics</th>
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<td>Variable</td>
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<td>Average (years)</td>
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<td>Follow-up</td>
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<td>Average (months)</td>
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<td>Chemotherapy</td>
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<td>Concomittant chemoradiotherapy (CCR)</td>
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<td>Recurrence</td>
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<tr>
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<td>Recurrence</td>
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<td>Primary</td>
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<td>Primary</td>
<td>Cordectomy + radiotherapy</td>
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<td>Primary</td>
<td>CCR</td>
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<td>Circular pharyngolaryngectomy + bilateral functional ND</td>
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<td>15</td>
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<td>T4N2M1</td>
<td>Recurrence</td>
<td>Laryngectomy + bilateral functional ND</td>
<td>Died free of the disease: 1 M</td>
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* The first patient (number 4) presented two synchronous primaries.
* The second (5) had a second primary of the lung.
that 42 (58%) of the 73 patients expressed galectin-3. A significant correlation was found between galectin-3 tumor positivity and longer relapse-free periods and overall survival. In univariate analysis high-grade (grade 3 or 4) tumors, non-keratinizing tumors and galectin-3-negative tumors showed a significantly increased risk of relapse and death. In multivariate analysis, only galectin-3 expression retained an independent prognostic significance for both relapse-free and overall survival, so demonstrating that the absence of galectin-3 expression is an independent negative prognostic marker in laryngeal squamous cell carcinoma patients. Plzak et al. showed that decreased levels of galectin-3 expression are associated with unfavorable prognoses in oropharyngeal and laryngeal cancer specimens. In the case of high stage hypopharyngeal squamous cell carcinomas, we observed that galectin-3 expression is not related to prognosis. Wu et al. analyzed the secreted proteomes of two nasopharyngeal cancer cell lines, with 23 proteins being found in cultured media from both lines. Of these proteins, fibronectin, galectin-3 and the plasminogen activator inhibitor 1 (PAI-1) were highly expressed in nasopharyngeal cancer biopsies, but only weakly, if at all, in normal nasopharyngeal tissue. The loss of galectin-3 expression in HNSCCs during the processes of dedifferentiation is a common feature in about all histological HNSCC types. Accordingly, we observed in the present study that the galectin-3 serum levels were significantly higher in the patients with metastatic HNSCCs than in those with localized HNSCCs. This feature also occurs in breast and gastrointestinal cancers.

Galectin-7 is overexpressed in buccal squamous cell carcinomas as compared to normal buccal epithelia. We determined the immunohistochemical expression of galectins-1, -3 and -7 in a series of 81 stage IV hypopharyngeal SCCs and observed that high levels of galectin-7 expression are associated with rapid recurrence rates and dismal prognoses, a feature not observed with galectin-3 and weakly, if at all, with galectin-1. These data suggest that the immunohistochemical determination of galectin-7 expression in the case of high-risk hypopharyngeal cancers is a meaningful tool to identify patients who should benefit from aggressive post-surgical adjuvant therapy.

The results of the present study indicate that patients with HNSCCs, even when they are associated with marked immunohistochemical levels of galectin-7, do not display galectin-7 in their blood. The reasons why only galectin-1 and galectin-3 can be detected in the serum to the exclusion of galectin-7 remain unknown. The mechanisms of galectin secretion is atypical and involves the formation of exosomes, vesicles that form on the outer surface of a cell as a result of membrane blebbing. However, there is no information describing exactly what causes galectin-1 and galectin-3 to accumulate at specific sites underneath the plasma membrane, and what actually causes the formation of exosomes when these proteins appear to be packaged in an active fashion.

**Conclusion**

The data from the present study show that the determination of circulating levels of galectin-1 and -3 in HNSCC
patients could be used to monitor the progression of their disease or their response to therapy. The galectin-3 concentrations in sera from the patients with a metastatic disease were higher than in sera from the patients with localized tumors. The levels of circulating galectin-1 were significantly decreased in a subset of HNSCC patients who underwent radiotherapy and chemotherapy or surgery and postoperative radiotherapy. We are now investigating (i) whether the decrease in galectin-1 serum expression in these patients parallels a disease-free and/or therapeutic control of the progression of the disease and (ii) whether increasing galectin-1 serum levels indicate a loss of therapeutic control in these patients. We are now further adding patients to our preliminary series of cases in order to arrive at a significant number of patients (200) to ensure reliable analyses. Once more, additional patients have to be added in the preliminary study before any definitive conclusions can be drawn.

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