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Galectin 7 (p53-Induced Gene 1): A New Prognostic Predictor of Recurrence and Survival in Stage IV Hypopharyngeal Cancer

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Background: Eighty percent of hypopharyngeal squamous cell carcinoma patients have advanced stages (III and IV) of the disease, and biological markers are required to predict high-risk head and neck squamous cell carcinoma patients in need of highly aggressive treatments after surgery to improve the survival rate. We analyzed the potential prognostic value of galectin 7 in a series of 81 stage IV hypopharyngeal SCCs because galectin 7 is an emerging marker involved in the epidermal development of pluristratified epithelia and in epidermal cell migration.

Methods: The immunohistochemical expression of galectin 7 was determined on a series of 81 stage IV hypopharyngeal SCCs and was compared with that of galectins 1 and 3.

Results: High levels of galectin 7 expression were associated with rapid recurrence rates and dismal prognoses in these 81 stage IV hypopharyngeal SCCs, a feature not observed with galectin 3 and one observed weakly, if at all, with galectin 1.

Conclusions: 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 postsurgical adjuvant therapy after surgery, including not only radiotherapy, but also chemotherapy.

Key Words: Galectins 1, 3, and 7—Hypopharyngeal cancer—Prognosis—Recurrence—Immunohistochemistry.

Head and neck squamous cell carcinomas (HNSCCs) are the sixth most common form of cancer worldwide[1] and constitute the most common malignant neoplasm arising in the mucosa of the upper aerodigestive tract.[2] The survival rates have not been improved during the past 30 years[1,2]: 50% of HNSCC

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patients die of their disease, and each year 5% develop additional primary tumors.[2] Furthermore, HNSCCs have a severe effect on the quality of life for both patients and survivors,[3,4] and, as emphasized by Hunter et al.,[1] the significant morbidity subsequent to treatment often mandates long-term multidisciplinary care, which leads to significant financial loads for treating institutions. Nearly two thirds of HNSCC patients present advanced stages (III and IV) of the disease, and, despite the use of resection and postoperative radiotherapy, high-risk (stage III and IV) HNSCCs frequently recur in the original tumor beds.[1–5] In this context, stage IV hypopharyngeal carcinomas represent one of the HNSCC subgroups associated with the worst prognosis, characterized by no more than 15% survival 5 years after diagnosis.

Biological markers are required to predict high-risk HNSCC patients in need of highly aggressive treatments consecutive to the surgical resection of tumors.[2,6,7] In recent studies, it has been demonstrated that galectins are involved in the development and progression of malignancies arising from a large set of tissue types.[8–10] On this basis, our objective was to quantitatively investigate the immunohistochemical expression of galectin 7 in stage IV hypopharyngeal SCCs and to determine whether the level of galectin expression could be associated with a good prognostic value for these advanced malignancies. Galectin 7 was chosen with regard to a large number of studies that have disclosed modifications of gene expression in HNSCCs by comparison with normal mucosa.[1,2,11] In particular, a strong correlation seemed evident between TP53 mutation and survival for HNSCC patients,[1] in whom the expression of galectin 7 is induced by PIG1 (p53-induced gene 1).[11,12] Galectin 7 is expressed in all stratified epithelia, and the onset of its expression coincides with the first visible signs of stratification.[11,13] Galectin 7 belongs to a family of growth/adhesive-regulatory lectins, i.e., galectins,[8–11] which share sequence homology and a set of invariant amino acids, such as Trp, the β-sandwich topology of folding, and Ca^{2+}-independent specificity to β-galactosides.[8–11,14] Currently, 15 different family members are known,[8–11] and galectin 1 and galectin 7, although they belong to the same subgroup of homodimeric galectins, present distinct effector functionalities.[8–11] The involvement of galectin 7 has already been evidenced in the growth regulation of SK-N-MC neuroblastoma cells (ATCC cell line, no. HTB 10).[11,15]

A series of reports has already emphasized the diagnostic and/or prognostic values of lectins in pathology.[16] In this context, galectins 1 and 3 were the most investigated galectins for the study of HNSCCs.[11,17–24] The first studies have demonstrated the expression of galectins 1 and 3 in a large number of HNSCCs in both cell lines and primary tumor specimens.[17,18] Additionally, it was shown that in HNSCCs tumors, the level of galectin 3 and galectin 3 binding site expression was correlated with the level of differentiation,[17,19,20] thus suggesting that the level of galectin 3 expression could be used as a prognostic factor for some types of HNSCCs.[21–24] Recently, Chen et al.[25] have performed a proteomic analysis of SCCs of the buccal mucosa to evidence tumor-associated proteins, among which emerged galectin 7. It is interesting to note that the homodimeric galectin 7 (p53-induced gene 1) is known to be a cell differentiation factor and a regulator of apoptosis functioning at the intracellular level through the Jun N-terminal kinase signaling pathway and the release of cytochrome *c*.[15,26,27] Altogether, these observations prompted us to study the potential prognostic value of galectin 7 by comparison with galectins 1 and 3 for a series of 81 cases of stage IV HNSCCs of hypopharyngeal origins. We have chosen to restrict our study to stage IV hypopharyngeal carcinomas obtained from a relatively homogeneous series of HNSCC patients to allow an accurate analysis of the actual prognostic value that could be attributed to galectins 1, 3, or 7.

MATERIALS AND METHODS

Histopathologic and Clinical Data

The hypopharyngeal squamous cell carcinoma (SCC) specimens were obtained from 5 female and 76 male patients who undergone radical surgery for curative purposes between January 1996 and December 2000 in the Ear, Nose, and Throat Department of the Hôpital Claude Huriez (Lille, France). The clinical staging was performed according to the tumor-node-metastasis classification system,[28] and the data relative to the 81 stage IV hypopharyngeal cancers are listed in Table 1. The diagnoses were established on the basis of histological criteria previously described by Hyams et al.[29] All hypopharyngeal SCCs involved in this study were primary tumors without metastases or recurrences and were categorized as well differentiated (n = 42), moderately differentiated (n = 28), and poorly differentiated (n = 11) tumors according to procedures detailed elsewhere.[19,21] More precisely, the well-differentiated lesions were characterized by the presence

TABLE 1. *Patient population characteristics*

Mean age, 55 y (range, 40–78 y)
Sex
Male: 76 patients (94%)
Female: 5 patients (6%)
Localization
Piriform sinus: 61 cases (75%)
Postcricoid area: 17 cases (21%)
Posterior wall: 3 cases (4%)
Grade
Well differentiated: 42 cases (52%)
Moderately differentiated: 28 cases (35%)
Poorly differentiated: 11 cases (13%)
TNM stage: 81 cases of stage IV
T2N2: 8 cases
T3N2: 8 cases
T4N0: 12 cases
T4N1: 7 cases
T4N2: 43 cases
T4N3: 3 cases
Treatment
Partial pharyngolaryngectomy: 9 cases
Total pharyngolaryngectomy: 54 cases
Circular pharyngolaryngectomy: 8 cases
Esopharyngolaryngectomy: 10 cases
29 had larynx cartilage invasion
118 neck dissections in 81 patients
49 patients with extranodal spread
Recurrence: 23 cases
Local recurrence: 17 cases
Distant recurrence: 11 cases
Follow-up
76 patients with clinical follow-up data
9 second primary tumors: 6 cases of lung cancer, 1 case of prostate cancer, and 1 case of kidney cancer
6 head and neck second primary tumors
29 deaths: 20 caused by the HNSCC and 9 without a direct relationship to the HNSCC (5 caused by a second primary tumor, 3 by medical diseases, and 1 of unknown origin)

HNSCC, head and neck squamous cell carcinoma.

of intercellular bridges and numerous foci of keratinization, whereas these structures were rarely observed in poorly differentiated tumors. The occurrence of single-cell keratinization and the presence of focal intercellular bridges were characteristic of a moderately differentiated phenotype.

All samples of hypopharyngeal SCCs used in this study came from patients who did not undergo chemotherapy or radiotherapy before surgery. After surgery, all patients were treated by standard radiotherapy but without chemotherapy. According to these criteria, the 81 specimens of stage IV hypopharyngeal SCCs that were investigated here correspond to a relatively homogeneous sample in terms of histopathologic and clinical criteria.

Immunohistochemistry

All specimens of hypopharyngeal SCCs were fixed for 24 hours in 4% formaldehyde, dehydrated, and routinely embedded in paraffin. Immunohistochemistry (detailed elsewhere[30–35]) was performed on 5-μm-thick sections on silane-coated glass slides. Briefly, before immunohistochemistry, dewaxed tissue sections were subjected to microwave pretreatment in .01 M citrate buffer (pH 6.0) for 2 × 5 minutes at 900 W. The sections were then incubated with .4% hydrogen peroxide for 5 minutes to block endogenous peroxidase activity, rinsed in phosphate-buffered saline (PBS; .04 M Na_2HPO_4, .01 M KH_2PO_4, and .12 M NaCl; pH 7.4), and exposed successively for 20 minutes to avidin (.1 mg/mL in PBS) and biotin (.1 mg/mL in PBS) to inactivate endogenous biotin. After rinsing in PBS, the sections were incubated for 20 minutes with .5% casein in PBS and exposed sequentially at room temperature to (1) the primary specific anti-galectin antibodies (see below), (2) the corresponding biotinylated secondary antibody (polyclonal goat anti-rabbit immunoglobulin G antibody or monoclonal mouse anti-goat immunoglobulin G antibody), or (3) the avidin-biotin-peroxidase complex (ABC kit). The presence of labeled peroxidase on the sections was visualized by incubation with a chromogenic substrate containing diaminobenzidine and hydrogen peroxide. After rinsing, the sections were counterstained with Luxol fast blue and mounted with a synthetic medium. For controls, the primary specific antibodies were omitted or replaced by nonimmune antisera. In all cases, these controls were negative. The biotinylated secondary antibodies and ABC kit came from DakoCytomation (Glostrup, Denmark).

Galectin 7 was immunolocalized in tumoral tissues with a polyclonal rabbit anti-human galectin 7 antibody, as detailed elsewhere.[34] The expression of recombinant human galectin 7, its purification by affinity chromatography, and the controls performed by gel electrophoresis and nanoelectrospray ionization mass spectrometry were detailed in previous articles.[15,30–32] Before immunohistochemistry, the polyclonal rabbit anti–galectin 7 antibody was tested by enzyme-linked immunosorbent assay and Western blotting to detect eventual cross-reactivity with other members of the three galectin subfamilies, i.e., galectins 1, 2, 3, 4, 8, and 9.[30–32] After these assays, a cross-reactivity observed for galectins 1 and 4 was removed by affinity depletion on resin bearing immobilized galectins.[30] The flow-through fraction was tested by the same procedures to ascertain the complete elimination of the cross-reactivity. The preparation of anti–galectin 1 and 3 antibodies and their controls has been detailed previously.[31]

For a series of 10 hypopharyngeal SCCs, we also immunolocalized galectin 7 by using a commercial

polyclonal goat anti-human galectin 7 antibody (R&D Systems, Minneapolis, MN). These immunolabelings were performed in parallel on serial sections for the two antibodies used in this study to compare the pattern of galectin 7 expression on tumoral tissues. The main objective of these comparisons was to determine the relative specificity of these antibodies and allow the eventual use of a commercially available antibody for routine pathology.

Computer-Assisted Microscopy

The levels of galectin expression after immunohistochemistry were quantitatively determined by using a computer-assisted KS 400 imaging system (Carl Zeiss Vision, Hallbergmoos, Germany). For each case, we scanned 15 fields corresponding to a total surface ranging from 60,000 to 120,000 μm^2. The analysis of the immunohistochemical expression of each marker by computer-assisted morphometry was quantitatively expressed by two variables: (1) the labeling index, which refers to the percentage of cells positively stained for a given marker, and (2) the mean optical density (MOD), which corresponds to the staining intensity of positive cells.[31,30,35]

Data Analysis

Numerical data obtained from independent groups were compared by the nonparametric Kruskal-Wallis test (more than two groups) or Mann-Whitney *U*-test (two groups). In contrast, categorical data, such as the localization of galectin expression, from independent groups were analyzed by using the χ^2 test or Fisher's exact test (in the 2×2 cases). Correlation between numerical variables was analyzed by means of the nonparametric Spearman correlation test.

The standard survival time analyses were performed by using Kaplan-Meier curves and the Gehan generalized Wilcoxon test. As previously described,[36] we have applied a decision tree–based technique to determine the threshold values needed to discriminate two groups of patients with very different clinical courses, such as deceased patients versus living patients without recurrence within a 24-month period after surgery. Briefly, for each variable of interest, this technique exhaustively investigates all the possible univariate splits between two observed values to identify the one that produces the greatest improvement in the process of distinguishing between the two groups of patients defined previously.[36] The selection of the best split from the set of possible candidate splits uses the Gini index, which is a measure of group mixture that reaches a value of 0 when discrimination is perfect (i.e., the two groups of interest are perfectly separated on the basis of the split selected). The statistical analysis was performed by using the software Statistica (StatSoft, Tulsa, OK).

RESULTS

Comparison of Anti–Galectin 7 Antibodies

The goal of this study was to investigate whether the immunohistochemical expression of galectin 7 could be used as a reliable prognostic factor for stage IV hypopharyngeal SCCs. This study thus relied on the use of an anti–galectin 7 antibody produced in our laboratory for which the distribution patterns of immunolabelings are detailed below. Additionally, as a control of our immunolabelings, but also to allow the eventual use of galectin 7 in routine pathology, we compared the data obtained with our specific anti–galectin 7 with those obtained by means of a commercially available anti–galectin 7 antibody. These comparisons were performed on serial sections obtained from 10 specimens selected at random out of the 81 hypopharyngeal SCC samples under study (see Materials and Methods). This comparative analysis showed that the distribution patterns of immunolabelings obtained for each of the anti–galectin 7 antibodies were nearly identical, as were the cellular locations of galectin 7 expression (data not shown). Similarly, a correlation analysis confirmed that the labeling index (the percentages of galectin 7–immunopositive cells) and the MOD (the immunohistochemical amounts of galectin–7 expression) obtained from quantitative morphometry were identical for the two antibodies (data not shown).

Immunohistochemical Expression of Galectin 7 in Stage IV Hypopharyngeal SCCs and Correlation With Clinical Features

Figure 1A and B illustrates the two types of patterns of galectin 7 immunohistochemical expression that we encountered in our hypopharyngeal SCC series. Although, as illustrated in Fig. 1A, galectin 7 expression was located in the cytoplasm in a large majority of cases (78% of the cases analyzed), several cases (22% of the cases analyzed) exhibited both cytoplasmic and nucleic expression, as illustrated in Fig. 1B. We thus investigated whether this latter group of cases showing double galectin 7 location could be statistically associated with particular clinical features. Our analysis

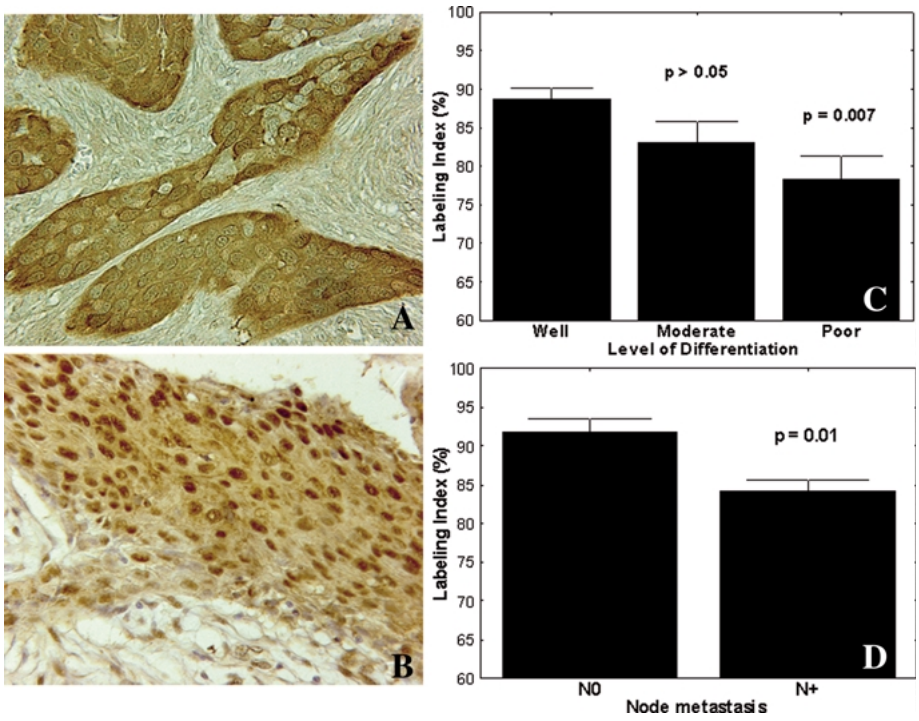

FIG. 1. (**A**) A case of head and neck squamous cell carcinoma (HNSCC) that exhibits galectin 7 expression exclusively localized in the cytoplasm (original magnification, ×400). (**B**) Another case showing a combination of cytoplasmic and nuclear labeling (original magnification, ×400). Variations of the percentage of immunopositive cells (labeling index) for galectin 7 in a series of 81 hypopharyngeal HNSCCs in relation to the differentiation grades (**C**) of the tumors (defined on the basis of histopathologic criteria; see Materials and Methods) or the presence (N^+) of nodal metastases (**D**) are shown. The data are expressed as mean ± SE. Statistical significance was evaluated by the Kruskal-Wallis test and (**C**) or (**D**) the Mann-Whitney test.

evidenced no significant association with any of the clinical features listed in Table 1 (i.e., the tumor location, the tumor differentiation status, the T status, and the presence/absence of nodal metastases). In contrast, the percentages of galectin 7–immunopositive cells significantly decreased in parallel with an apparent loss of histological differentiation (Fig. 1C; Kruskal-Wallis test; $P = .004$) and with the presence of nodal metastases (Fig. 1D; Mann-Whitney test; $P = .01$). No significant variation was observed in the immunohistochemical amounts of galectin 7 expression (data not shown).

We also observed weak but significant correlations between the percentages of galectin 7–immunopositive cells and those of galectin 1 (Spearman $r = .30$; $P = .007$) and galectin 3 (Spearman $r = .38$; $P = .003$) and, to a lesser extent, between the amounts of galectin 7 and those of galectin 1 (Spearman $r = .24$; $P = .03$) and galectin 3 (Spearman $r = .29$; $P = .02$). In contrast, no significant correlation was evidenced between galectin 1 and galectin 3 expression.

Selection of the HNSCC Samples

Previous reports (see the introduction) have described a parallelism between the decrease of galectin 3 expression in HNSCCs, independent of their histological origin, and the loss of differentiation. We therefore used this characteristic as a tool to investigate whether our series of 81 hypopharyngeal SCCs were similar to the cases described in the literature. The data illustrated in Fig. 2 clearly indicate for stage IV hypopharyngeal SCCs that both the percentages of galectin 3–immunopositive cells (Fig. 2A) and the immunohistochemical amounts of galectin 3 expression (Fig. 2B) decreased in parallel with an apparent loss of histological differentiation.

The pattern of immunohistochemical galectin 3 expression is illustrated in Fig. 2C for a well-differentiated hypopharyngeal SCC and in Fig. 2D for a poorly differentiated tumor. Fig. 2E illustrates the distribution of the 81 hypopharyngeal SCCs under investigation in relation to the percentages of galectin 3–immunopositive SCC cells (y-axis) and to the MOD (x-axis) in relation to the levels of differentiation. Altogether, the data reported in Fig. 2 suggest that no major caveat was introduced in the course of our analysis, a fact that strengthens the potential prognostic value of galectin 7 expression in stage IV hypopharyngeal SCCs (see below).

Contribution of Galectins 1, 3, and 7 to the Prognosis of Hypopharyngeal HNSCC Recurrence

In this study, we have made the distinction between deceased patients and patients whose tumors did not recur during a 2-year period after surgery. This selection was made on the basis of a decision tree

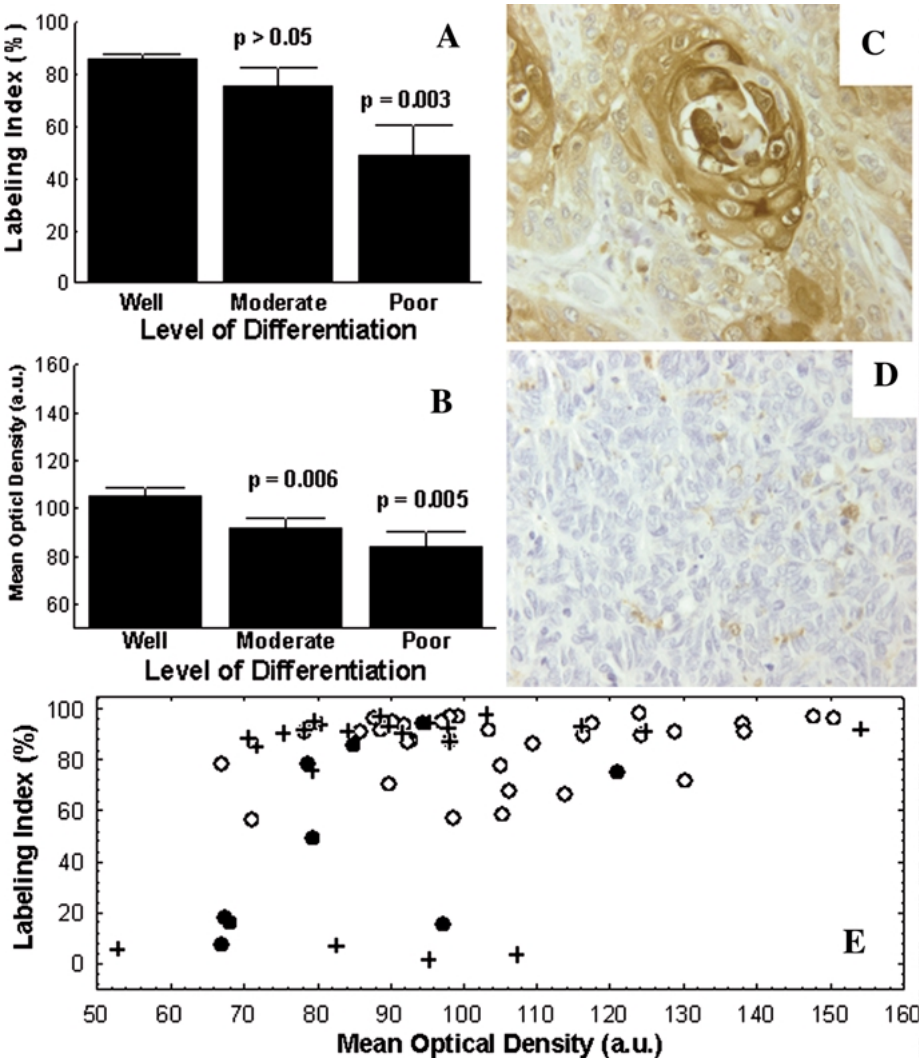

FIG. 2. Labeling index (**A**) and mean optical density (**B**) from a series of 81 hypopharyngeal head and neck squamous cell carcinomas (HNSCCs) after immunostaining with anti−galectin 3 antibody. The three grades of the tumors were defined on the basis of histopathologic criteria (see Materials and Methods). The data are expressed as mean ± SE. Statistical significance was evaluated by the Mann-Whitney *U* test by comparison with the group of well-differentiated tumors. Expression of immunoreactive galectin 3 is shown in a well-differentiated (**C**) or in a poorly differentiated (**D**) hypopharyngeal HNSCC. The foci of keratinization are clearly positive in well-differentiated tumors, whereas immunostaining appears diffuse in poorly differentiated HNSCC (original magnification, ×200). (**E**) Comparative distribution of galectin 3−immunopositive HNSCC cells on the basis of the labeling index (y-axis) and the mean optical density (x-axis). Each symbol refers to an individual value in relation to the stage of differentiation of the tumor (black circles, poor; +, moderate; white circles, differentiated). a.u., arbitrary unit.

technique that allowed the definition of a statistically significant threshold that was based on the amount of galectin 7 expression for each case of hypopharyngeal SCC (see Materials and Methods). This threshold, corresponding to 134 arbitrary units for our hypopharyngeal SCC series (Fig. 3A), allowed discrimination between patients with recurrence (Fig. 3D) and between patients who showed a significant difference in terms of their periods of survival after surgery (Fig. 4). The same procedure was applied for galectins 1 and 3. However, in the case of galectin 3, no threshold value was considered as useful by the decision tree technique to discriminate between deceased patients and patients whose tumors did not recur during a 2-year period after surgery (Fig. 3B). By means of Kaplan-Meier analyses performed on several threshold values "manually" selected, we verified that the amount of galectin 3 expression did not provide prognostic value in terms of hypopharyngeal SCC recurrence (data not shown). In the case of galectin 1, we obtained a weak but statistically significant threshold value at 115 MOD arbitrary units (Fig. 3C). In contrast, the threshold value obtained for galectin 7 was associated with a clear-cut prognostic value in terms of hypopharyngeal SCC recurrence, which reached highly significant values ($P < .001$) when considering the 51 patients with periods of recurrence >12 months (data not shown). In fact, Fig. 3D illustrates that the prognostic value obtained with galectin 7 remained applicable for very long periods after surgery, at least >16 months (45 patients). The high value of statistical significance ($P < .001$) in the case of the galectin 7 remained stable for periods of recurrence >24 months (32 patients) and was thus not modified when the number of hypopharyngeal SCC patients involved in the analysis decreased. In contrast, a similar analysis shows that the amount of galectin 1 presented only a transient and

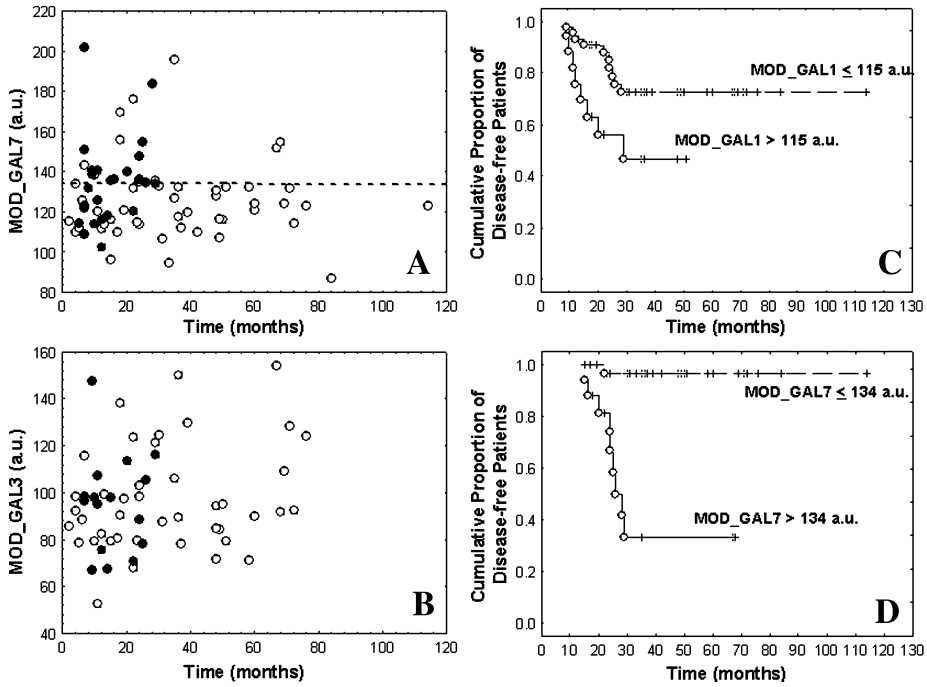

FIG. 3. (**A**) Comparative distribution of galectin 7–immunopositive head and neck squamous cell carcinoma cells on the basis of the mean optical density (y-axis; ordinate) and the follow-up of the patients (x-axis). Each symbol refers to an individual value in relation to the recurrence status of the patients (white circles, patients with no recurrence; black circles, patients with recurrence). The horizontal dashed line corresponds to a threshold value (mean optical density [MOD] of 134 a.u.) obtained on the basis of a decision tree drawn for discriminating between deceased patients and patients without recurrence who were still alive 24 months after surgery. Similarly, (**B**) shows the distribution obtained in the case of galectin 3 for which the decision tree technique did not determine any discriminatory threshold. In (**C**) and (**D**), crosses correspond to patients with no tumor recurrence, whereas dots indicate patients with tumor recurrence; (**C**) illustrates the remission curves obtained in the case of patients with no recurrence 8 months after surgery and associated with the two groups of patients distinguished on the basis of quantitative morphometry of immunoreactive galectin 1 in tumoral tissue (below or above the value of 115 a.u. for the MOD). (**D**) Remission curves obtained in the case of patients with no recurrence 16 months after surgery and associated with the two groups of patients distinguished on the basis of quantitative morphometry of immunoreactive galectin 7 tumor tissue (below or above the value of 134 a.u. for the MOD). a.u., arbitrary unit.

weak prognostic value in terms of hypopharyngeal SCC recurrence, i.e., only in the case of considering the 61 patients with periods of recurrence >8 months (Fig. 3C). In conclusion, the quantitative evaluation of galectin 7 thus seems to be a robust method to estimate the recurrence rates of high-stage hypopharyngeal SCC. Finally, the analysis of the amount of galectin 3 expression seemed irrelevant to the study of hypopharyngeal SCC, at least with respect to the evaluation of recurrence rates (Fig. 3B).

Contribution of Galectins 1, 3, and 7 to the Prognosis of Survival Consecutive to Hypopharyngeal HNSCC

To estimate the potential prognostic value of galectins 1, 3, and 7 in terms of patient survival after hypopharyngeal SCC, we have used the protocol previously described for the study of hypopharyngeal SCC recurrence rates (described previously). Similar to recurrence analysis, our analysis showed that galectin 7 was again associated with a clear-cut prognostic value in terms of periods of survival for hypopharyngeal SCC patients. Figure 4B illustrates the prognostic value associated with the quantitative determination of galectin 7 in hypopharyngeal SCC patients with survival periods >16 months ($n = 51$). Similarly significant P values ($P < .01$) were obtained when we considered patients with survival periods longer than 8, 12, 16, or 20 months and were even improved ($P < .001$) when we considered patients with longer survival periods (data not shown). Some of these patients (arrows in Fig. 4B) developed a secondary cancer diagnosed during the clinical follow-up consecutive to the primary hypopharyngeal SCC. Nevertheless, in these circumstances, the prognostic value associated with the quantitative determination of galectin 7 (data not shown) was not significantly modified ($P > .05$).

In contrast with the data recorded for galectin 7, the observations performed with galectin 1 immunohistochemistry remained associated with a low prognostic value when we considered patients with survival periods >16 months (Fig. 4A). Furthermore, in contrast

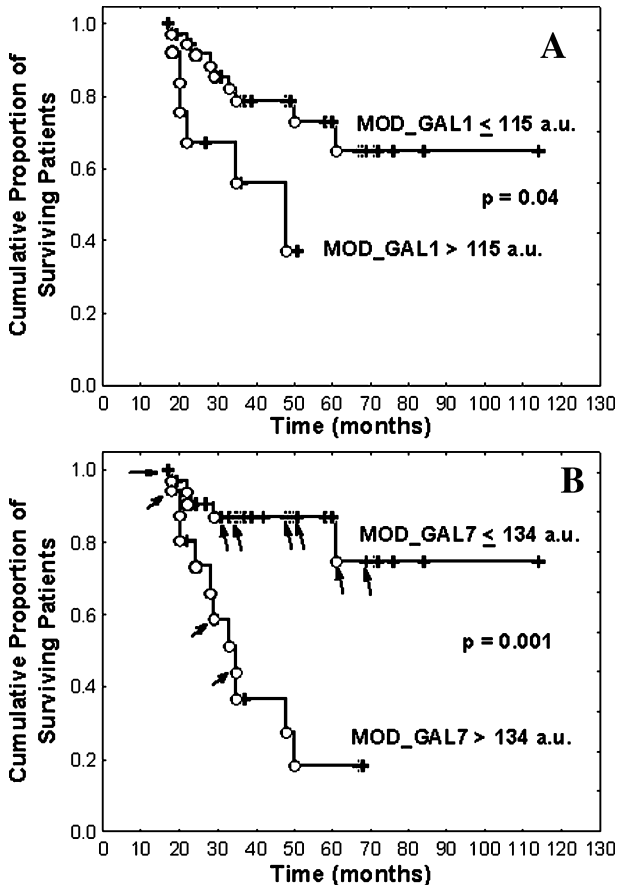

FIG. 4. Similar to Figs. 3C and D, (**A**) and (**B**) show the survival curves associated with the two groups of patients determined on the basis of the threshold values used in Fig. 3C and D who did not die during the first 16 months after surgery. The dead and living patients are indicated by means of circles and crosses, respectively. In (**B**), the arrows identify the patients who showed at least one other tumor during follow-up. MOD, mean optical density. a.u., arbitrary unit.

to galectin 7, this prognostic value associated with galectin 1 did not remain significant ($P > .05$) when the other category of patients was considered (i.e., by adding patients with shorter survival periods or restricting to patients with longer survival periods; data not shown). As illustrated in Fig. 3B, no discriminant threshold was available for the amount of galectin 3 expression in HNSCC. As in the case of recurrence (described previously), manual value selection confirmed that this quantitative variable did not disclose a significant prognostic value in terms of survival periods for patients after hypopharyngeal SCC.

DISCUSSION

This study indicates that the immunohistochemical level of galectin 7 expression in high-stage hypopharyngeal SCCs correlates highly with tumor recurrence rates, a feature that could explain why the immunohistochemical levels of galectin 7 expression relate to patient survival. This feature is far less pronounced with galectin 1 and not at all with galectin 3. This observation is in sharp contrast with the study by Piantelli et al.,[23] who showed that a significant correlation was found between galectin 3 tumor positivity and longer disease-free and overall survival and that the absence of galectin 3 expression is an independent negative prognostic marker in HNSCCs. The discrepancy between our study and the one reported by Piantelli et al.[23] is easily explainable as follows. Our study relies on the analysis of 81 stage IV hypopharyngeal SCCs, whereas the one by Piantelli et al.[23] is based on the analysis of 73 node-negative laryngeal HNSCC patients. We had reported previously that the glycobiological characteristics of laryngeal cancers, including the levels of expression of galectin 3 and galectin 3 binding sites, were very different in laryngeal tumors as compared with the other remaining types of HNSCC tumors.[21] This point relates directly to the fact that cancers of the upper aerodigestive tract are heterogeneous in their neoplastic processes, each of which requires its own unique set of epidemiological, anatomical, pathologic, and therapeutic considerations.[21] It must also be emphasized that HNSCCs have different embryological origins: some of them arise in the ectoderm and others in the endoderm.[5]

It is interesting to note that in this study and previous[19,21] studies, as in the ones by Piantelli et al.[23] and other groups,[17,24] the levels of expression of galectin 3 decreased as the levels of differentiation decreased in all types of HNSCCs, whereas, as shown here, galectin 3 expression is not associated with any prognostic value in the case of hypopharyngeal cancers. These data suggest that the histological level of differentiation, as defined on the basis of keratinization levels, is not a reliable marker of malignancy, at least in the case of hypopharyngeal SCCs.

This study shows that an increasing level of galectin 7 is associated with negative prognoses in the case of hypopharyngeal SCCs. At first glance, the findings of our work are somewhat counterintuitive and at odds with the findings of other investigators. Indeed, galectin 7 expression is induced by p53 and functions as a regulator of differentiation and apoptosis.[11–13,26,27] Galectin 7 should therefore aid in eliminating tumor cells,[11,27,37] and an increasing level of galectin 7 should therefore be theoretically associated with a favorable prognosis—a feature definitely not observed in this study. However, our

findings can be explained, at least partly, by those very recently reported by Demers et al.,[38] whose study also attributed the existence of a previously undescribed activity, the promotion of cancer cell malignancy, to galectin 7. These authors[38] found that the development of experimental thymic lymphoma is accelerated when induced by lymphoma cells overexpressing galectin 7. They provide data suggesting that galectin 7 modulates the aggressive behavior of lymphoma cells by controlling the expression of metastatic genes, such as metalloproteinases (MMPs), including MMP-9.[38] The involvement of MMPs in general, and MMP-9 in particular, in the aggressive behavior of HNSCCs is already well documented.[39,40] We are currently investigating by means of small interfering RNA techniques whether the modification of galectin 7 expression in human pharyngeal carcinoma cell lines leads to distinct patterns of development and/or metastatic potential when they are grafted onto immunodeficient mice, in relation to the levels of expression of MMP-9.

In conclusion, the study by Piantelli et al.[23] clearly showed that the histochemical detection of galectin-3 in laryngeal patients could be useful in the establishment of adjuvant therapy protocols for the selection of node-negative patients with potentially unfavorable outcomes. The data from our study define galectin-7 as a marker for the identification of stage IV hypopharyngeal cancer patients with worse prognoses who might be candidates for more aggressive therapy. Indeed, two studies recently demonstrated that postoperative concurrent administration of high-dose cisplatin with radiotherapy was more efficacious than radiotherapy alone in locally advanced (stage III and IV) head and neck cancers.[3,4]

The results of this study can, at least partly, also be explained in light of those reported by Lu et al.[41] and Moisan et al.[42] First, Lu et al.[41] showed that galectin 7 is overexpressed in rat mammary carcinomas induced by 7,12-dimethylbenz[a]anthracene. Thus, overexpression of galectin 7 is in direct relation to the increased biological aggressiveness in experimental tumors. Second, to ascertain the complex pattern of gene expression involved in the evolution of aggressiveness in lymphoma cells, Moisan et al.[42] compared the transcriptome of 164T2 lymphoma cells with that of their aggressive variants. These authors thus identified several genes that were differentially expressed in nonmetastatic lymphoma cells and their metastatic variants.[42] Galectin 7 was one of the gene products whose expression was significantly upregulated in metastatic variants, a result similar to the increased levels of immunohistochemical expression of galectin 7 observed here in the most aggressive hypopharyngeal HNSCCs. The explanation given by Moisan et al.[42] of the involvement of galectin 7 in the metastatic processes of lymphoma cells is as follows. On the basis of work with p53-dependent apoptosis onset in DLD-1 colon cancer cells, galectin 7 is 1 of the 14 of the 7002 genes tested whose expression is induced in the early steps of p53-mediated apoptosis.[12] DNA methylation of several genes has previously been associated with the transformation of normal cells into tumor cells or the progression from a nonmetastatic phenotype to a metastatic one.[43,44] In some instances, DNA methylation of a single CpG dinucleotide is sufficient to control the expression of p53.[45] Although galectin 7 tissue distribution is very narrow (present mostly in all stratified epithelia), an examination of the human galectin 7 promoter reveals a strong transcriptional potential conferred by the presence of three repeats of the GGGTGG motifs present in the 5′-flanking sequence.[41,42] The specificity of tissue expression of galectin 7 suggests that its expression is controlled by strong suppressive factors, a feature in which DNA methylation may play a major role. Indeed, the data provided by Moisan et al.[42] show that the treatment of lymphoma cells with 5-aza-CdR, an agent that modulates the levels of DNA methylation, contributes to the upregulation of galectin 7. In reference to a work published by Kuwabara et al.,[26] who show that stable transfectants of HeLa cells expressing galectin 7 exhibit enhanced sensitivity to apoptosis while expressing a large set of other genes, Moisan et al.[42] hypothesized that highly metastatic variants of lymphoma cells overexpressing galectin 7 have evolved toward a state of resistance to the proapoptotic function of galectin 7, whereas their aggressiveness has emerged from the galectin 7 function itself and/or from genes induced by its presence.

Finally, to avoid the problems with reproducibility that confound studies with certain galectins because of the use of different reagents,[46] we deliberately ascertained that our antibody preparation yielded results comparable to the data obtained with use of a commercially available product.

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REFERENCES

1. Hunter KD, Parkinson EK, Harrison PR. Profiling early head and neck cancer. *Nat Rev Cancer* 2005; 5:127–35.
2. Grandis JR, Pietenpol JA, Greeberger JS, et al. Head and neck cancer: meeting summary and research opportunities. *Cancer Res* 2004; 64:8126–9.
3. Cooper JS, Pajak TF, Forastiere AA, et al. Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck. *N Engl J Med* 2004; 350:1937–44.
4. Bernier J, Domenge C, Ozsahin M, et al. Postoperative irradiation with or without concomitant chemotherapy for locally advanced head and neck cancer. *N Engl J Med* 2004; 350:1945–52.
5. Schantz SP, Harrison LB, Forastiere AA. Tumors of the nasal cavity and paranasal sinuses, nasopharynx, oral cavity, and oropharynx. In: De Vita VT Jr, Hellman S, Rosenberg SA, eds. Cancer: *Principles and Practice of Oncology*. Vol 1, 5th ed. Philadelphia: Lippincott, 1997; 741–847.
6. Shah JP. *Head and Neck Surgery*. 2nd ed. New York: Mosby-Wolfe, 1996.
7. Myers JN. The use of biological therapy in cancer of the head and neck. *Curr Probl Cancer* 1999; 23:106–34.
8. Liu FT, Rabinovich GA. Galectins as modulators of tumor progression. *Nat Rev Cancer* 2005; 5:29–41.
9. Gabius HJ, Siebert HC, André S, et al. Chemical biology of the sugar code. *Chembiochem* 2004; 5:740–64.
10. Danguy A, Camby I, Kiss R. Galectins and cancer. *Biochim Biophys Acta* 2002; 1572:285–93.
11. Saussez S, Kiss R. Galectin-7. Review. *Cell Mol Life Sci* 2006; 63:686–97.
12. Polyak K, Xia Y, Zweier JL, et al. A model for p53-induced apoptosis. *Nature* 1997; 389:300–5.
13. Timmons PM, Colnot C, Cail I, et al. Expression of galectin-7 during epithelial development coincides with the onset of stratification. *Int J Dev Biol* 1999; 43:229–35.
14. Lahm H, André S, Hoeflich A, et al. Tumor galectinology: insight into the complex network of a family of endogenous lectins. *Glycoconj J* 2004; 20:227–38.
15. Kopitz J, André S, von Reitzenstein C, et al. Homodimeric galectin-7 (p53-induced gene 1) is a negative growth regulator for human neuroblastoma cells. *Oncogene* 2003; 22:6277–88.
16. Saussez S, Marchant H, Nagy N, et al. Quantitative glycohistochemistry defines new prognostic markers for cancers of the oral cavity. *Cancer* 1998; 82:252–60.
17. Gillenwater A, Xu XC, El-Naggar AK, et al. Expression of galectins in head and neck squamous cell carcinoma. *Head Neck* 1996; 18:422–32.
18. Gillenwater A, Xu XC, Estrov Y, et al. Modulation of galectin-1 content in human head and neck squamous carcinoma cells by sodium butyrate. *Int J Cancer* 1998; 75:217–24.
19. Delorge S, Saussez S, Pelc P, et al. Correlation of galectin-3/galectin-3-binding sites with low differentiation status in head and neck squamous cell carcinomas. *Otolaryngol Head Neck Surg* 2000; 122:834–41.
20. Plzak J, Smetana K Jr, Hrdlickova E, et al. Expression of galectin-3 reactive ligands in squamous cancer and normal epithelial cells as a marker of differentiation. *Int J Oncol* 2001; 19:59–64.
21. Choufani G, Nagy N, Saussez S, et al. The levels of expression of galectin-1, galectin-3, and the Thomsen-Friedenreich antigen and their binding sites decrease as clinical aggressiveness increases in head and neck cancers. *Cancer* 1999; 86:2353–63.
22. Honjo Y, Inohara H, Akahani S, et al. Expression of cytoplasmic galectin-3 as a prognostic marker in tongue carcinoma. *Clin Cancer Res* 2000; 6:4635–40.
23. Piantelli M, Iacobelli S, Almaddri G, et al. Lack of expression of galectin-3 is associated with a poor outcome in node-negative patients with laryngeal squamous-cell carcinoma. *J Clin Oncol* 2002; 20:3850–6.
24. Plzak J, Betka J, Smetana K Jr, et al. Galectin-3—an emerging prognostic indicator in advanced head and neck carcinoma. *Eur J Cancer* 2004; 40:2324–30.
25. Chen J, He QY, Yuen APW, et al. Proteomics of buccal squamous cell carcinoma: the involvement of multiple pathways in tumorigenesis. *Proteomics* 2004; 4:2465–75.
26. Kuwabara I, Kuwabara Y, Yang RY, et al. Galectin-7 (PIG1) exhibits pro-apoptotic function through JNK activation and mitochondrial cytochrome C release. *J Biol Chem* 2002; 277:3487–97.
27. Ueda S, Kuwabara I, Liu FT. Suppression of tumor growth by galectin-7 gene transfer. *Cancer Res* 2004; 64:5672–6.
28. Sobin LH, Wittekin DC. *TNM Classification of Malignant Tumours UICC International Union Against Cancer*. 6th ed. West Sussex, UK: Wiley BR, 2002.
29. Hyams VJ, Batsakis JG, Michaels L, eds. *Tumors of the Upper Respiratory Tract and Ear. Atlas of Tumor Pathology*. Washington, DC: Armed Forces Institute of Pathology, 1988.
30. Gabius HJ. Influence of type of linkage and spacer on the interaction of β-galactoside-binding proteins with immobilized affinity ligands. *Anal Biochem* 1990; 189:91–4.
31. André S, Kojima S, Yamazaki N, et al. Galectins-1 and -3 and their ligands in tumor biology. *J Cancer Res Clin Oncol* 1999; 125:461–74.
32. André S, Pieters RJ, Vrasidas I, et al. Wedgelike glycodendrimers as inhibitors of binding of mammalian galectins to glycoproteins, lactose maxiclusters, and cell surface glycoconjugates. *Chembiochem* 2001; 2:822–30.
33. Camby I, Belot N, Rorive S, et al. Galectins are differentially expressed in supratentorial pilocytic astrocytomas, astrocytomas, anaplastic astrocytomas and glioblastomas, and significantly modulate tumor astrocyte migration. *Brain Pathol* 2001; 11:12–26.
34. Rorive S, Eddafali B, Fernandez S, et al. Changes in galectin-7 and cytokeratin-19 expression during the progression of malignancy in thyroid tumors: diagnostic and biological implications. *Mod Pathol* 2002; 15:1294–301.
35. Nagy N, Legendre H, Engels O, et al. Refined prognostic evaluation in colon carcinoma using immunohistochemical galectin fingerprinting. *Cancer* 2003; 97:1849–58.
36. Decaestecker C, Camby I, Remmelink M, et al. Decision tree induction: a useful tool for assisted diagnosis and prognosis in tumor pathology. *Lab Invest* 1997; 76:799–808.
37. Bernerd F, Sarasin A, Magnaldo T. Galectin-7 overexpression is associated with the apoptotic process in UVB-induced sunburn keratinocytes. *Proc Natl Acad Sci USA* 1999; 96:11329–34.
38. Demers M, Magnaldo T, St-Pierre Y. A novel function for galectin-7: promoting tumorigenesis by up-regulating MMP-9 gene expression. *Cancer Res* 2005; 65:5205–10.
39. O-Charoenrat P, Wongkajornsilp A, Rhys-Evans PH, et al. Signaling pathways required for matrix metalloproteinases-9 induction by betacellulin in head-and-neck squamous carcinoma cells. *Int J Cancer* 2004; 111:174–83.
40. Wiegand S, Dunne AA, Muller HH, et al. Metaanalysis of the significance of matrix metalloproteinases for lymph node disease in patients with head and neck squamous cell carcinoma. *Cancer* 2005; 104:94–100.
41. Lu J, Pei H, Kaeck M, et al. Gene expression changes associated with chemically induced rat mammary carcinogenesis. *Mol Carcinogenesis* 1997; 20:204–15.

42. Moisan S, Demers M, Mercier J, et al. Upregulation of galectin-7 in murine lymphoma is associated with progression toward an aggressive phenotype. *Leukemia* 2003; 17:751–9.
43. Laird PW, Jaenisch R. The role of DNA methylation in cancer genetic and epigenetics. *Annu Rev Genet* 1996; 30:441–64.
44. Baylin SB, Herman JG. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends Genet* 2000; 16:168–74.
45. Pogribny IP, Pogribna M, Hristman JK, et al. Single-site methylation within the p53 promoter region reduces gene expression in a reporter gene construct: possible in vivo relevance during tumorigenesis. *Cancer Res* 2000; 60: 588–94.
46. Itzkowitz SH. Galectins: multipurpose carbohydrate-binding proteins implicated in tumor biology. *Gastroenterology* 1997; 113:2003–5.