Changes in Galectin-7 and Cytokeratin-19 Expression during the Progression of Malignancy in Thyroid Tumors: Diagnostic and Biological Implications

Sandrine Rorive, M.D., Brahim Eddafali, M.D., Sergio Fernandez, M.D., Christine Decaestecker, Ph.D., Sabine André, Ph.D., Herbert Kaltner, Vet.M.D, Ichiro Kuwabara, Ph.D., Fu-Tong Liu, Ph.D., Hans-Joachim Gabius, Ph.D., Robert Kiss, Ph.D., Isabelle Salmon, M.D., Ph.D.

Laboratory of Pathology, Cliniques Universitaires de Bruxelles, Hôpital Erasme (SR, BE, SF, IS), and Laboratory of Histopathology, Faculty of Medicine, Université Libre de Bruxelles (CD, RK), Brussels, Belgium; Institute of Physiological Chemistry, Faculty of Veterinary Medicine, Ludwig-Maximilians-University (SA, HK, H-JG), Munich, Germany; and Department of Dermatology, School of Medicine, University of California, Davis (IK, F-TL), Sacramento, California

Galectin-7 is associated with p53-dependent onset of apoptosis and proliferation control/differentiation in keratinocyte development. It is also up-regulated in chemically induced rat mammary carcinogenesis. Because the levels of expression of galectin-7 have never been investigated in thyroid tumors (in contrast to those of galectin-1 and -3 associated with malignancy), we initiated analysis of the expression of galectin-7 in benign and malignant thyroid lesions together with that of cytokeratin-19 (CK19), a marker already demonstrated to be useful in diagnosing this kind of lesion. The immunohistochemical expression levels were quantitatively determined by means of computer-assisted microscopy on a series of 84 thyroid lesions including 10 multinodular goiters, 32 adenomas, and 42 carcinomas. Our data clearly indicate a marked down-regulation of galectin-7 expression in a large proportion of adenomas (including the normomacrofollicular, microfollicular, and trabecular variants) if compared with carcinomas. In accordance with results of previous studies, a marked up-regulation of CK19 expression was observed in the thyroid carcinomas, and this contrasted in particular with the low CK19 expression observed in the microfollicular adenomas. Of importance for diagnostic implications, the combination of these two markers enabled our series of microfollicular adenomas (characterized by low galectin-7 and CK19 expression) to be efficiently distinguished from the encapsulated follicular variant of papillary thyroid carcinomas (high galectin-7 and CK19 expression).

KEY WORDS: Adenoma, Carcinoma, Cytokeratin-19, Diagnosis, Galectin-7, Thyroid, Quantitative immunohistochemistry.

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The immunohistochemical monitoring of factors involved in growth control and/or cell adhesion can uncover determinants of signaling/effector pathways relevant for malignant progression and thus diagnostic purposes. In this sense, the definition of new protein families with respective properties *in vitro* guides the selection of potential markers for histopathological monitoring. Based on the concept of information transfer from glycans of cellular glycoconjugates to endogenous receptors (lectins) and supported by the changes of glycosylation seen on malignant transformation (1–3), the analysis of tissue lectins in cancer may constitute a new class of tumor markers. Focusing on lectins with specificity to β-galactosides, the family of galectins (13 members currently are known in mammals) deserves attention. Indeed, various family members have been documented to be involved in the mentioned fundamental processes, and recent monitoring of expression by means of RT-PCR or a panel of galectin-type-specific antibodies has revealed that tumor cells can well express several activities at the same time (4–9). This situation leads to questions

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Address reprint requests to: Isabelle Salmon, M.D., Ph.D., Laboratory of Pathology, Erasmus Hospital, 808 route de Lennik, B-1070 Brussels, Belgium; e-mail: isalmon@ulb.ac.be; fax: 322-555-4790.

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as to whether a galectin could be linked to tumor parameters and, next, to what extent the presence of more than one galectin might influence such a conclusion. Instructive examples on the second part of the question have only recently been provided by showing functional divergence of galectin-1 and -3 *in vitro* in neuroblastoma cells and the suppressor-like expression pattern of galectin-8 in malignancy development of colon (10, 11). In general, galectin-1 and -3 primarily have been studied with respect to diagnostic and prognostic values associated with their levels of expression in tumor pathologies in general (9, 12–14), and in thyroid tumors in particular (15–24). In the Discussion for this article, we detail the current opinion about the actual diagnostic and prognostic information contributed by galectin-1 and especially galectin-3 in thyroid tumor pathologies. These studies point to a diagnostic value associated with galectin-3 expression in thyroid tumors, especially either an absent or low level of expression in benign thyroid tissues as opposed to high levels in malignant tissues (17–24). Variable staining deviating from the general pattern in adenomas and carcinomas, however, deserve attention for reaching a general conclusion (25, 26). With the emerging insights into functional aspects of further family members, it is imperative to address the issue to extend these initial studies with galectin type–specific reagents. For reasons outlined in the following paragraph, we followed this concept and focused on galectin-7.

The level of expression of galectin-7 has so far not been investigated, at least to our knowledge, in thyroid tumors. We quantified its presence in 84 thyroid lesions, including 42 benign and 42 malignant cases. The cDNA for galectin-7 was independently cloned in the search for markers defining the normal keratinocyte (27, 28). The first studies published on galectin-7 suggested its role in epidermal differentiation processes (27–30). Presence in bladder cancer (31) and up-regulation during chemical carcinogenesis of rat mammary tissue (32) appear to contrast with its role associated with p53-induced apoptosis in the DLD-1 colon carcinoma cell line (33) and keratinocytes exposed to UVB irradiation (34), an aspect crucial in referring to galectin-7 as *PIG1* (p53-induced gene 1; 35). As a pro-apoptotic protein, galectin-7 appears to function intracellularly in HeLa cells upstream of JNK activation and cytochrome C release (35). Thus, it is of interest to investigate systematically whether galectin-7 is present in thyroid cancer. In view of the indications for a role of galectin-3 in this tumor class, the question will thus be answered on the relation between galectin-7 presence and disease progression. To exclude the possibility of a nonrepresentative case selection, we added the concomitant analysis with a marker with known and well-characterized staining control as internal control (see below).

In contrast to the case of galectin-7, the expression of cytokeratin-19 (CK19) has already been investigated in thyroid lesions, revealing promising characteristics for diagnosis (36–38), which should yet be viewed with caution (39). CK19 is the lowest molecular weight keratin (40 kDa) and is widely present in simple epithelial cells. Its expression is focal or restricted to basal cells in complex and stratified epithelia. As shown for example by Miettinen *et al.* (36), CK19 is expressed differentially in various types of thyroid lesions in which a malignant transformation is generally paralleled by an increased level of CK19 expression. CK19 immunoreactivity is not specific to malignancy, however. In fact, the extent and intensity of staining must be considered to exhibit different patterns of expression in relation to benign lesions and their malignant counterparts (36, 40).

The aim of the present study was to quantitatively determine the immunohistochemical expression of galectin-7 and CK19 in a series of 84 thyroid lesions, including 10 multinodular goiters, 32 adenomas, and 42 carcinomas. This quantitative determination was carried out by means of computer-assisted microscopy as detailed previously for thyroid (22) and brain (40, 41) tumors.

MATERIALS AND METHODS

Histopathological Diagnoses

In the present study we applied the diagnostic criteria that we had already followed in previous studies (22, 42, 43), namely the histological criteria proposed by the World Health Organization (WHO) classification of thyroid tumors (44). The benign group included 10 multinodular goiters (MNGs) and 32 adenomas (ADs). Adenomas exhibit various architectural patterns; four categories are standardly described, including the trabecular/solid subtype (without follicles and in which the cells grow in a diffuse way), the microfollicular subtype (showing neoplastic follicles smaller than the follicles of the normal thyroid gland), the normofollicular subtype (with neoplastic follicles similar in size than the follicles of the normal gland), and the macrofollicular subtype (exhibiting large follicles similar to those in hyperplastic nodules). An admixture of the latter two subtypes (labeled *normomacrofollicular*) is also commonly seen (22, 42, 43, 45, 46). Our series of adenomas included normomacrofollicular (MAC_AD: $n = 8$), microfollicular (MIC_AD; $n = 19$), or trabecular (TRA_AD; $n = 5$) patterns.

The cancers ($n = 42$) included papillary carcinomas (PAP; $n = 17$) and their follicular variants (PAP-

_FOL; $n = 9$), follicular (FOL; $n = 5$), and anaplastic (ANA; $n = 6$) carcinomas. Of the follicular variants of the papillary carcinomas, five were encapsulated. In addition, the present study also included five medullary (MED) carcinomas.

Quantitative Immunohistochemistry

All 84 specimens were fixed in 4% formaldehyde and embedded in paraffin; 5-μm-thick sections were processed with a polyclonal anti-galectin-7 antibody and second-step kit reagents under study. Recombinant expression of human galectin-7 used a pQE-60/hGal-7 plasmid in *E. coli* M15[pREP4] cells (35, 47). After a polyclonal antibody fraction was raised in a rabbit, it was tested by ELISA and Western blotting against representative members of each of the three galectin subfamilies, that is, galectins 1, 3, 4, and 8. Also, FACScan analysis with galectin-7–negative cells expressing other galectins on the cell surface was performed. Cross-reactivity was detected for galectins 1 and 4. To remove this cross-reacting activity, we performed two cycles of affinity depletion by chromatography on the respective resin-immobilized galectins attached to Sepharose 4B after activation of the matrix by divinyl sulfone as described elsewhere (48). Reexamination of the flow-through fraction indicated complete removal of the contaminating activity. When comparing galectin-7 and galectin-3 immunoreactivity in different tissue samples, we observed galectin-3 and galectin-7 immunopositivity in blood vessels, whereas lymphocytes did not express these two galectins (data not shown). A different pattern was observed in fibrosis areas, which exhibited galectin-7 positivity and galectin-3 negativity (data not shown).

The mouse monoclonal antibody against CK19 was provided by Novocastra Laboratories (Newcastle upon Tyne, UK).

The extent of immunoreactivity was visualized by means of avidin-biotin-peroxidase complex (ABC) kit reagents (Vector Labs, Burlingame, CA), with diaminobenzidine/H_2O_2 as the chromogenic substrates; this process is described elsewhere (22, 40, 41). The omission of the incubation step with the antibody served to exclude any staining caused by the binding of the kit reagents such as mannose-rich glycoproteins, that is, horseradish peroxidase and avidin. The use of a control serum instead of the antibody fractions excluded non-antigen-dependent binding. Counterstaining with hematoxylin concluded the processing. Figure 1 provides

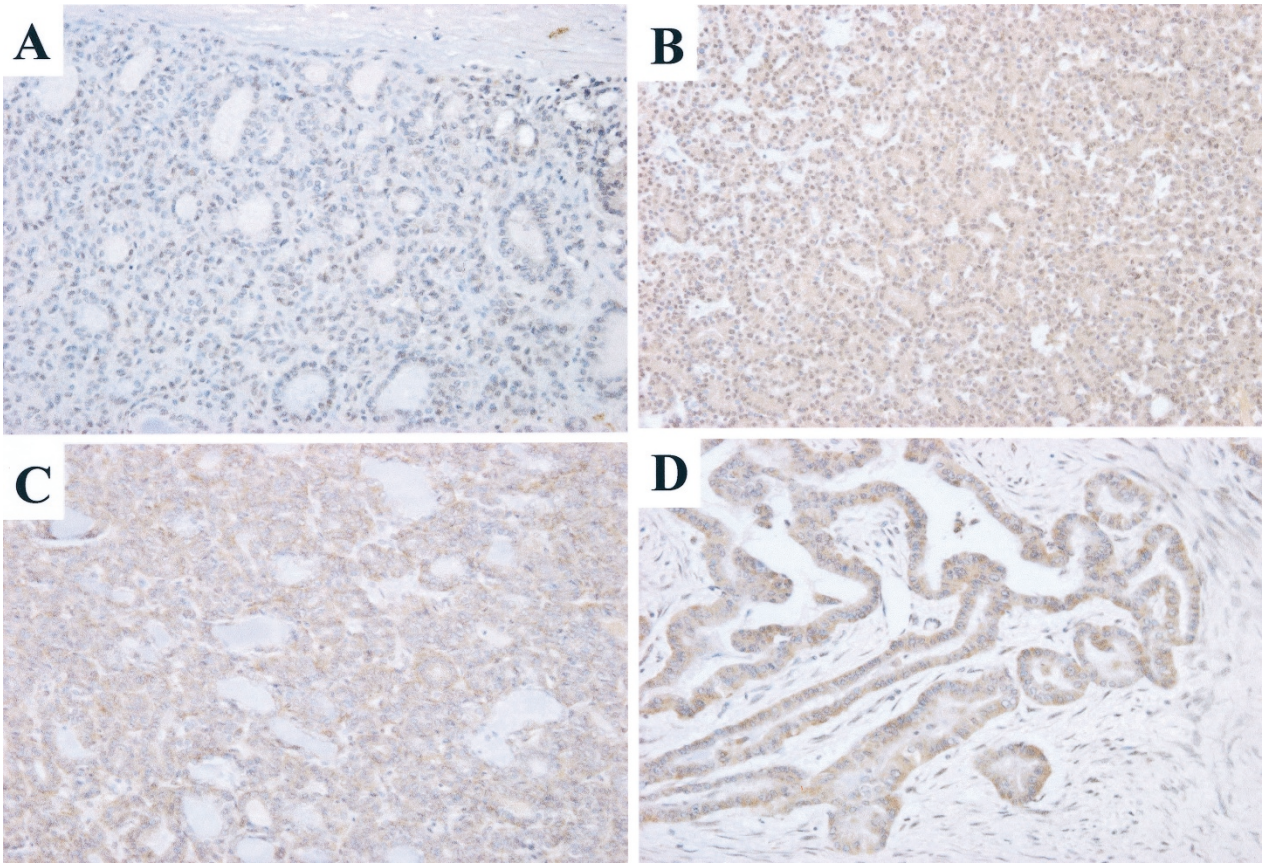

FIGURE 1. Morphological illustrations (100×) of immunohistochemical galectin-7 expression in a microfollicular adenoma (**A**), a follicular carcinoma (**B**), a follicular variant of a papillary carcinoma (**C**), and a papillary carcinoma (**D**).

exemplary morphological illustrations of staining patterns for galectin-7 in benign and malignant thyroid tumors.

The levels of immunohistochemical expression were quantitatively determined by means of a SAMBA 2005 computer-assisted microscope system (Samba Technologies, Grenoble, France) with a 40× (Olympus BX50 microscope, aperture 0.65) magnification lens. Ten fields of between 60,000 and 120,000 μm^2 each were scanned for each case. The computer-assisted system used to quantify the staining and the data processing is detailed elsewhere (22, 40, 41). Briefly, this system provides two quantitative variables, that is, the labeling index (LI), which refers to the percentage of cells specifically stained by a given histochemical marker, and the mean optical density (MOD), which denotes staining intensity.

Statistical Analysis

The associations between the categorical variables were tested by means of the Fisher exact test or the χ^2 test, depending on whether the two variables analyzed were binary or not. Mann-Whitney nonparametric tests were also used to compare groups of numerical data (because the conditions of application of the standard Student *t* tests were not verified). All of the statistical analyses were performed with Statistica/Windows software (StatSoft, Tulsa, OK).

RESULTS

Immunohistochemical Galectin-7 Expression in Thyroid Lesions

Figure 1 illustrates the patterns of immunohistochemical galectin-7 expression in a microfollicular adenoma (Fig. 1A), a follicular carcinoma (Fig. 1B), a follicular variant of a papillary carcinoma (Fig. 1C), and a papillary carcinoma (Fig. 1D). This figure shows that galectin-7 patterns of expression varied across these different cases. We therefore made use of computer-assisted microscopy to quantify the individual levels of galectin-7 expression in benign and malignant thyroid tissue.

Figure 2A (individual values) and Figure 2B (mean values) show that nearly the entire cell population in the multinodular goiters (the MNG group) expressed galectin-7, as indicated by LI values close to 100%. In contrast, the percentage of immunohistochemically galectin-7–positive cells in the thyroid adenomas (the AD group) and the carcinomas (the CAN group) varied markedly from one case to another and ranged between 5 and 100%, depending on the cases analyzed. Whereas the galectin-7–dependent staining intensity remained

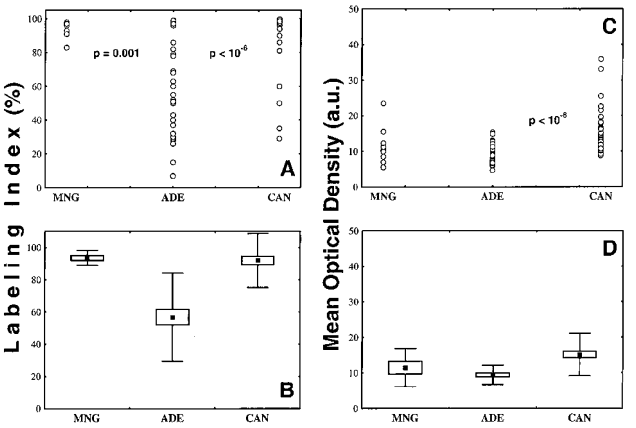

FIGURE 2. Illustration of the extent of galectin-7 expression and the differences between the three groups of thyroid lesions, namely multinodular goiters (MNG), adenomas (ADE), and carcinomas (CAN). The data are reported in terms of the labeling index (**A–B**; quantifying the percentage of immunopositive cells) and the mean optical density (**C–D**; quantifying the immunostaining intensity in arbitrary units). Although (**A**) and (**C**) show data for the individual cases (with possible overlaps between the dots), (**B**) and (**D**) show the mean values (***small squares***) with their SE (***boxes***) and SD (***bars***), where *P* values are given for any significant difference.

similar ($P > .05$) in the multinodular goiters and the adenomas (Fig. 2C–D), it was significantly higher ($P < 10^{-6}$) in the carcinomas than in the adenomas (Fig. 2C–D).

The course of galectin-7 expression followed a bell-shaped down- and up-regulation (in terms of galectin-7–immunopositive thyroid cells) in the sequence multinodular goiters → normomacrofollicular adenomas → microfollicular adenomas → trabecular adenomas → papillary carcinomas → follicular variant of papillary carcinomas → follicular carcinomas, with a minimum of galectin-7–immunopositive cells observed in the microfollicular group of adenomas (Fig. 3A–B). The staining intensity for galectin-7 remained similar ($P > .05$) across all the groups under study (Fig. 3C–D).

Immunohistochemical CK19 Expression in Thyroid Lesions

Figure 4A–B shows that the carcinomas (the CAN group) exhibited a significantly larger number of CK19-positive cells than did the other two groups ($P < 10^{-6}$), whereas the staining intensity remained similar ($P > .05$) across the three groups (Figs. 2C and 2D). Detailing these results, with the exception of the medullary subgroup, Figure 5A–B shows a strong increase in terms of the percentage of CK19-immunopositive cells in all the carcinoma subgroups when compared with the case of benign lesions including the multinodular goiters (MNG), the normomacrofollicular adenomas (MAC_AD), and the microfollicular adenomas (MIC_AD). On the basis of these observations, the trabecular adenomas (TRA_AD) and the medullary carcinomas

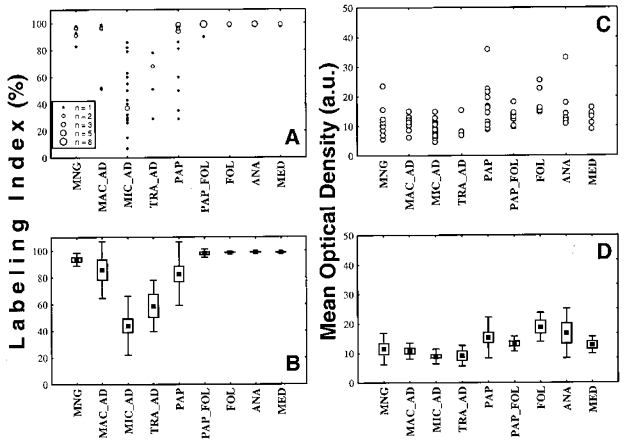

FIGURE 3. Details of the data reported in Figure 2 in terms of the nine different subgroups of thyroid lesions included in our series, that is, multinodular goiters (MNGs), normomacrofollicular (MAC_AD), microfollicular (MIC_AD), and trabecular (TRA_AD) adenomas, papillary carcinomas (PAP) and their follicular variants (PAP_FOL), and follicular (FOL), anaplastic (ANA), and medullary (MED) carcinomas. The rest of the legend is identical to that of Figure 2.

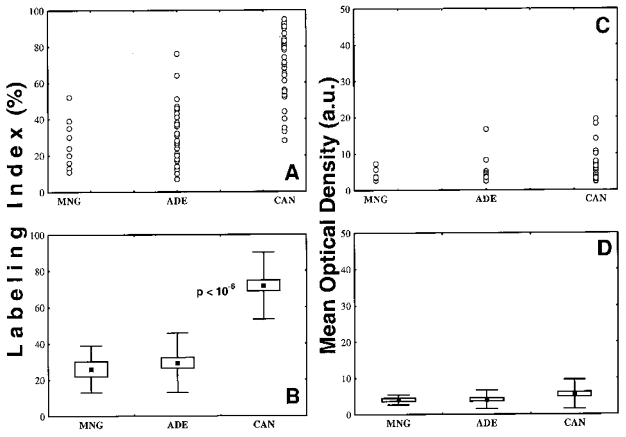

FIGURE 4. Similar to the case in Figure 2, Figure 4 illustrates the differences in CK19 expression across the three groups of thyroid lesions. The rest of the legend is identical to that of Figure 2.

(MED) exhibited an intermediary level of expression. Concerning the staining intensity for CK19 (Fig. 5C–D), the papillary carcinomas exhibited significantly stronger staining than that of any other type of lesion (with *P* values for significance of $<.01$).

Differential Expression of Galectin-7 and CK19 between the Microfollicular Adenomas and the Encapsulated Follicular Variants of the Papillary Thyroid Carcinomas

Figure 6A and B shows that when compared with the microfollicular adenomas, both galectin-7 and CK19 are significantly up-regulated (in terms of the percentage of immunopositive cells) in the six encapsulated follicular variants of papillary thyroid carcinomas present in our series. Furthermore, the combination of these two markers enabled these

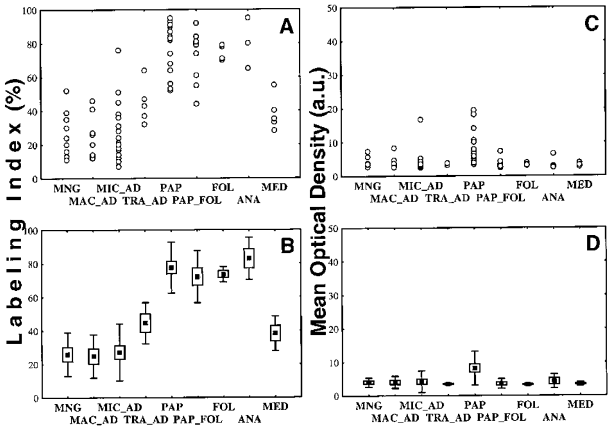

FIGURE 5. Details of the data reported in Figure 4 in terms of the nine different subgroups of thyroid lesions included in our series. The rest of the legend is identical to that of Figure 3.

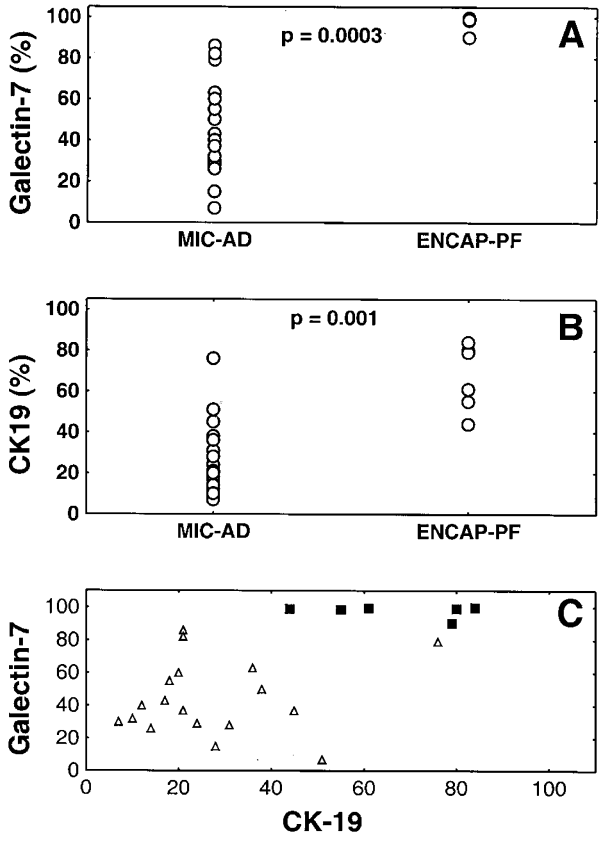

FIGURE 6. Illustration of the differences in galectin-7 (**A**) and CK19 (**B**) expression (in terms of the labeling index) between the microfollicular adenomas (MIC-ADE) and the encapsulated follicular variants of papillary thyroid carcinoma (ENCAP-PF). **C,** the data distribution (MIC-ADE as triangles and ENCAP-PF as squares) for these two markers.

two groups of lesions to be efficiently distinguished from one another (Fig. 6C).

DISCUSSION

As stated by LiVolsi (45), the level of malignancy in thyroid cancers ranges from that of almost be-

nign papillary carcinomas to that of anaplastic carcinomas, one of the most aggressive carcinomas in humans. Several subgroups can also be identified in the group of thyroid adenomas that in themselves are benign tumors. Problems in thyroid tumor diagnosis occur between microfollicular adenomas and minimally invasive follicular carcinomas. There are reliable histological criteria for diagnosing patients with minimally invasive follicular carcinomas of the thyroid gland: these criteria include cellularity, capsularity, capsular invasion, vascular invasion, extension into the parenchyma, cytoplasmic oxyphilia, mitotic activity, and necrosis (49). Many of these criteria require the surgical excision of the tumor for histopathological examination, and most of them cannot therefore be used on cytological samples obtained from fine needle aspiration biopsy (FNAB; 50).

Several groups of researchers have identified galectin-3 as a marker for distinguishing benign from malignant follicular thyroid tumors. Gasbarri *et al.* (20) observed that normal thyrocytes do not express galectin-3 and that galectin-3 is never expressed in benign thyroid lesions, whereas it is invariably detected in thyroid cancers. Saggiorato *et al.* (23) observed only 4 of 52 microfollicular adenomas expressing galectin-3 immunopositivity, whereas all thyroid cancers that those investigators analyzed were immunopositive for galectin-3. In the same way, Orlandi *et al.* (18) reported that although all the thyroid cancers that they analyzed were galectin-3 immunopositive, only 3 of 29 thyroid adenomas exhibited such positivity. Those authors (18) claimed that although those three cases had been diagnosed as benign on the basis of histopathological criteria, they were inclined to consider them to be true follicular carcinomas (because of their galectin-3 immunopositivity), in which it is not possible to identify capsular and/or vascular invasion. These data have led several groups of researchers (18–20, 23, 24) to use the immunodetection of galectin-3 on cytological specimens obtained from FNABs as a supposedly accurate method for selecting on a molecular basis those thyroid lesions that need to be surgically resected.

Intertumoral heterogeneity, noted in several studies (25, 26), yet calls for a cautious interpretation of results on galectin-3 expression, especially regarding recommendation for further treatment. Interestingly, some cases that are referred to as benign exhibit galectin-3 immunopositivity. Considering the data listed above, absence of galectin-3 expression in thyroid adenomas and its strong presence in malignant cases intimates that the respective microfollicular adenomas are in the process of malignant transformation. This proposal can also be discussed with respect to the evaluation of further tumor features. We have already carried out a number of studies to address the issue on a malignant transformation in certain follicular adenomas of the thyroid gland. We used computer-assisted microscopy analyses of Feulgen-stained nuclei in a series of 238 thyroid lesions with the aim of quantitatively describing morphometric (nuclear area) and textural (chromatin pattern) morphonuclear characteristics (42), and nuclear DNA content (43). These two studies suggest a preneoplastic nature of microfollicular adenomas. We reached the same conclusions by the identification of distinct glycan epitopes in thyroid adenomas, as opposed to thyroid carcinomas (22), and by the analysis of the influence of various growth factors and hormones on the cell proliferation of human thyroid tumors (51). These data, with conspicuously very different experimental designs, clearly suggest the preneoplastic nature of a set of thyroid adenomas that exhibit microfollicular histopathological characteristics. Focusing on the insights that our studies obtained into the galectin network in this tumor class, the profile of galectin-7 expression is different from that of galectin-1 and -3. Normal tissue and adenomas were devoid of these two galectins, whose presence thus appeared to be associated with the malignant transformation (15, 16). Interestingly, nuclear transcription assays in oncogene-transformed cell lines (v-myc, v-raf, K-ras) for galectin-1 and down-regulation of galectin-3 in cells of the thyroid papillary line NPA support this notion (52, 53). The percentage of galectin-7–immunopositive cells (not the staining intensity), however, was subject to down-regulation in the adenoma group. The first lesson that therefore emerges is that of differential regulation, especially of the prototype galectin-1 and -7. It thus seems likely that their activity profiles in this tumor type will not completely overlap. The most pronounced change in galectin-7 positivity was apparent between cell populations in normomacrofollicular, relative to microfollicular, adenomas, with a drastic decrease. At this crucial step, reduced galectin-7 expression could help cells to escape the onset of apoptosis, considering that this protein expression is controlled by p53 in DLD-1 colon cancer cells and keratinocytes (33, 34) and is associated with proliferation control and differentiation in the development of embryonic keratinocytes and in squamous cell carcinomas (29, 30, 34). Having proceeded further to malignancy, galectin-7 might assume roles characteristic of the malignant phenotype, as reflected by its up-regulation in chemically induced rat mammary carcinogenesis (32). This interpretation intimates a dual role of galectin-7 at different stages in the course of thyroid cancer development. That the same galectin can be associated with pro- and antitumoral characteristics has recently been documented for

galectin-1 and neuroblastoma *versus* glioma cells (10, 40, 41, 54–56). In sensitive cells, this galectin can induce apoptosis and nonapoptotic cell death but also tumor growth stimulation and transformation (4, 8, 10, 57–60). Thus, the second emerging lesson on galectin-7 expression is that its expression profile in thyroid lesions can be reconciled with a dual role exerted at different stages in the pathway from benign to malignant tumors. Further studies using manipulation of expression *in vitro* will be helpful to support its interpretation.

The results on evaluation of the combination of the markers (galectin-7 and CK19) deserve a comment. The present data clearly indicate a marked down-regulation of galectin-7 expression in a large proportion of the microfollicular adenomas, with such down-regulation also observed in some of the normomacrofollicular and trabecular adenomas and in a small number of the papillary carcinomas (Fig. 3). In accordance with results of previous studies (for example, 36–40), our data also revealed a marked up-regulation of CK19 expression in the thyroid carcinomas (except the medullary ones), a factor that contrasts in particular with the low CK19 expression observed in the microfollicular adenomas (Fig. 4). Consequently, the combination of these two markers enabled our series of microfollicular adenomas (low expression of both galectin-7 and CK19) to be clearly distinguished from the encapsulated follicular variant of papillary thyroid carcinomas (the high expression of both galectin-7 and CK19; *cf* Fig. 6).

In conclusion, our series of analyzed thyroid specimens documents an advantage of combining CK19 and galectin-7 expression to separate thyroid carcinomas from adenomas, in particular, the encapsulated follicular variant of papillary thyroid carcinomas from microfollicular adenomas.

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