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Galectin-3: Presurgical marker of thyroid follicular epithelial cell-derived carcinomas

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ABSTRACT. Preoperative follicular lesion characterisation represents an unsolved diagnostic problem in thyroid nodular disease. Although fine-needle aspiration biopsy is the most reliable preoperative diagnostic procedure, it shows inherent limitations in differentiating adenoma from follicular carcinoma and, sometimes, follicular variants of papillary carcinoma. Galectin-3 cytoplasmic neoexpression has been proposed as a peculiar feature of thyroid malignant cells, easily detectable in cytological and histological samples. The aim of this study was to re-evaluate the galectin-3 expression in a large sample of thyroid lesions using an immunohistocytochemical biotin-free detection system and a specific anti-human-galectin-3 monoclonal antibody in order to avoid the interference of technical factors, a cause of conflicting results recently reported by some authors. We analysed galectin-3 expression of 39 follicular carcinomas, 26 papillary carcinomas, and 105 adenomas in both cell-block samples and their histological counterparts. All cell-block and histological papillary carcinoma samples showed high levels of galectin-3 immunoreactivity. Thirty-four follicular carcinomas were positive, whereas 5 were negative in cell-blocks but positive in their histological counterparts. Twelve out of 105 adenomas expressed galectin-3 in cell-blocks and histological samples. The diagnostic accuracy of preoperative galectin-3 evaluation in adenomas *vs* follicular carcinomas was 90.0%. Galectin-3 expression was also investigated in 22 minimally-invasive follicular carcinomas. All of them showed galectin-3 immunoreactivity in both cytological and histological specimens with the exception of two cases, where galectin-3 positivity was observed only in the surgical material. The routine correct use of galectin-3, by increasing the diagnostic accuracy of conventional cytology, improves the management of thyroid nodules and can lead to a sensitive reduction of useless thyroid surgeries.

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INTRODUCTION

In thyroid nodular disease, an unsolved diagnostic problem is represented by the preoperative characterisation of follicular lesions. The diagnostic approach to "thyroid nodule" is usually based on fine-needle aspiration biopsy (FNAB), a well-established complementary procedure to the clinical, ultrasound and scintigraphic examinations (1, 2). The large consent encountered by FNAB is due to its inherent features of easiness, reproducibility, accuracy and cheapness. FNAB assessments allow a more appropriate characterisation of thyroid nodules, and a more accurate selection of patients requiring surgery (1-4). However, the most important FNAB limitation is represented by the lack of sensitivity in the evaluation of follicular neoplasms, due to its inability to detect the tumor capsule and vessel infiltrations. Less than 25% of the nodules with indeterminate FNAB results ("follicular neoplasm") show malignant features at histological examination (about 6% of all FNABs). Thus, about 80% of the total FNABs with indeterminate results are histologically represented by benign lesions, corresponding to unnecessary surgical operations (5). In addition, a frequent cause of cytological and histological misdiagnoses is represented by minimally invasive carcinomas (MICs), which account for 50% of all follicular cancers. Because of their cy-

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tomorphological features overlapping those of follicular adenomas, except for minimal vascular and/or capsular invasions (6-8), MICs are a serious challenge in the presurgical assessment of thyroid nodules.
Although several additional techniques, such as ultrasonography (9, 10), computed image processing systems (11) and large-needle biopsy (12), have been developed to increase the FNAB diagnostic accuracy for thyroid lesions, at the moment none of them has led to a real significant improvement in solving the "follicular neoplasm" dilemma (13). Therefore, the identification of reliable molecular markers for thyroid malignant cells, easily and cheaply detectable by immunocytochemical methods, is of paramount importance in order to provide new morphological criteria for an early presurgical diagnosis, and to avoid the "indeterminate" diagnostic definition. To this purpose, recent advances in molecular diagnostics, such as immunocytochemistry, enzyme activity assays, and RT-PCR, may be useful in improving the FNAB accuracy, allowing further and better analyses of molecular products from biopsies. Several molecules (at least 50) involved in carcinogenic processes have been proposed as markers of thyroid malignancy (oncogene products, altered enzymes, integrins, cadherins, lectins, etc.) (14-16). Among them, telomerase (17, 18), HMGI(Y) (19), HBME-1 (20, 21), immunologically altered thyroid peroxidase (TPO) (22, 23), which is not recognised by MoAb47 monoclonal antibody, and galectin-3 (24) seem to be the most promising molecules in significantly increasing the FNAB sensitivity in follicular lesions.
Galectin-3 polypeptide is a member of the oligosaccharide-selective-binding protein family known as lectins (25). Galectin-3 plays important roles in cell-cell and cell-matrix interactions (26), extracellular matrix organisation (27), mRNA splicing (28), cell growth and apoptosis (29), neoplastic transformation and metastatisation (30, 31). As reported by some studies, this lectin is expressed in thyroid carcinoma but not in normal thyrocytes and in benign lesions such as follicular adenoma (32, 33). Moreover, its malignancy predictive value has been assessed also in cytological samples (34, 35).
Nevertheless, result discrepancies have been recently reported (36-38). Some of these disagreements have been ascribed to the employment of RT-PCR techniques (39), immunohistochemical biotin-detection systems (40), and/or monoclonal antibodies against non-human galectin-3 but cross-reacting with the human one (40). In particular, the use of biotin-based methods and non-species-specific monoclonal antibodies can potentially result in misinterpretations, due to the interference of follicular cell non-specific antigenicity (40, 41).

The aim of this study was to re-evaluate in a biotin-free detection system, using a commercially available mouse anti-human galectin-3 monoclonal antibody, the accuracy of galectin-3 test in a large sample of thyroid lesions, and the lectin ability to correctly characterise MIC.

MATERIALS AND METHODS

Subjects

A total of 170 consecutive surgical specimens and their corresponding cytological counterparts, collected from patients who had undergone surgery at San Luigi Hospital, Orbassano (Turin) between 1989 and 2003 due to nodular thyroid disease with histological diagnosis of follicular thyroid carcinoma or adenoma, were retrospectively studied (6). Part of the cases (62%) came from our archive and had already been tested for galectin-3, using a biotin-based detection system. The remaining 38% were first tested in this study. Euthyroid status, negative calcitonin serum levels, cytological and histological material availability, and preoperative FNAB results of "follicular neoplasm" or of malignancy were the criteria required for the enrolment of patients in the study. Patients consisted of 26 males and 144 females, with a median age of 44.6 yr (range 16-78). All of them had one or more palpable thyroid nodules; in the latter case, the nodule was selected for FNAB assessment on the basis of suggestive ultrasonographic features of malignancy (10). A preoperative FNAB cytological result of "follicular neoplasm" was given in 151 out of 170 cases (105 follicular adenomas; 39 follicular carcinomas; 6 follicular and 1 tall-cell variants of papillary carcinoma), whereas malignancy was reported in 19 papillary carcinomas. Malignancy was clinically suspected in only three widely invasive follicular carcinoma cases (2 trabecular and 1 insular follicular subtypes), due to the presence of hard and fixed nodule with ipsilateral lymphadenopathy, and ultrasonographic features of hypoechogenicity, irregular margin, invasive growth, and regional lymphadenopathy. Patients received a final histological diagnosis of adenoma (105 cases), papillary carcinoma (26 cases - 14 classical types, 9 follicular variants, 3 tall-cell variants), and follicular carcinoma (39 cases - 10 well-differentiated carcinomas, 19 Hürthle-cell carcinomas, 7 trabecular and 3 insular poorly differentiated carcinomas). Among follicular carcinomas, 22 were defined as minimally invasive (7 well-differentiated carcinomas, 11 Hürthle-cell carcinomas, and 4 trabecular carcinomas) due to the histological finding of minimal full thickness capsular invasion and/or infiltration of less than 4 blood vessels located within or immediately outside the capsule (6). This study was approved by the San Luigi Hospital committee of medical ethics, and all the patients gave written consent.

FNABs

FNABs were performed with a 22-gauge x 1.5-in needle attached to a 30-ml plastic syringe. After aspiration, a small amount of fluid was expelled from the needle, and smeared in part onto poly-L-lysine-coated slides, fixed, and stained using the Papanicolaou method for a rapid specimen adequacy assessment. FNAB specimen adequacy was defined in the presence of at least six groups of ten preserved follicular cells. The remaining material was used for cell block preparation, in which galectin-3 expression was subsequently evaluated by the immunoperoxidase technique.

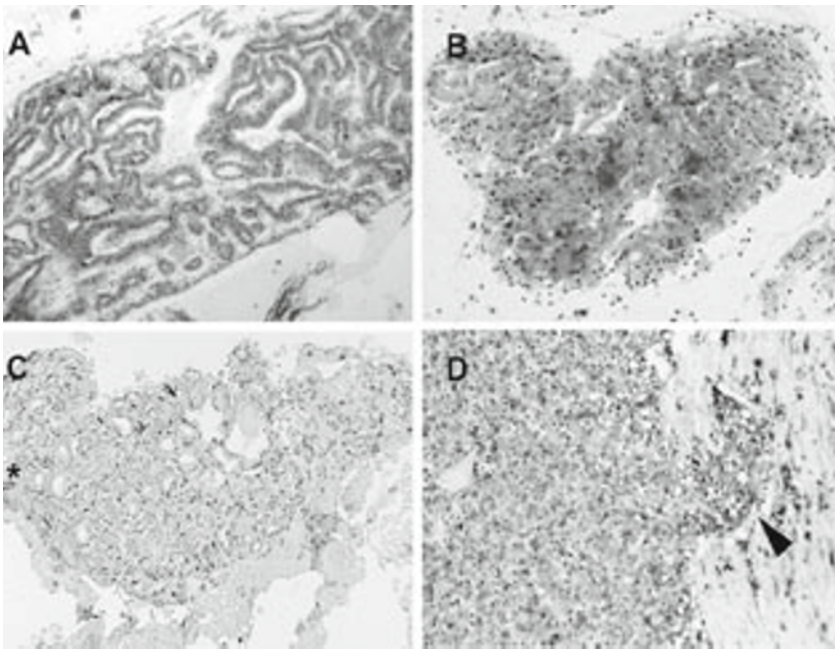

Fig. 1 - *Galectin-3 expression in surgical and cytological samples of thyroid follicular-derived neoplasms. A. Papillary carcinoma. Cytoplasmic immunoreactivity. (Streptavidin-biotin immunoperoxidase with Mayer's solution counterstain. Magnification x160). B. Hürthle cell carcinoma. Cytoplasmic immunoreactivity. (Streptavidin-biotin immunoperoxidase with Mayer's solution counterstain. Magnification x160). C. Follicular adenoma. Nuclear labelling of sporadic neoplastic cells, without cytoplasmic immunoreactivity. The asterisk marks a vessel with positivity confined to the endothelial cells. (Streptavidin-biotin immunoperoxidase with Mayer's solution counterstain. Magnification x160). D. Follicular adenoma with galectin-3 immunoreactivity (false positive). Cytoplasmic and cytoplasmic/nuclear immunopositivity. The arrow shows minimal infiltration of the capsule, with no signs of entire thickness capsular penetration. (Streptavidin-biotin immunoperoxidase with Mayer's solution counterstain. Magnification x160). No evidence of vascular or capsular invasion was found in multiple histological serial sections.*

Cytological specimens

Cell-blocks were obtained by fixing the aspirated fragments into an alcoholic solution (alcohol 95°) for 2h at room temperature and centrifuging for 10 min at 3000 r/min. After an additional 3-h incubation in 95° alcohol, the sedimented specimens were dehydrated and paraffin-embedded.

Surgical specimens

The surgical specimens were formalin fixed and paraffin-embedded for both routine histopathological examination and immunohistochemical staining.

Immunoperoxidase technique

Galectin-3 immunostaining was evaluated in both 4-µm-thick cytological and histological sections collected onto poly-L-lysine-coated slides with a standard manual streptavidin-peroxidase procedure using a biotin-free detection system (Envision System, Dako, Glostrup, Denmark). Human galectin-3 was revealed using a mouse monoclonal antibody (clone 9C4, diluted 1:500; Novocastra Laboratories Ltd, Newcastle, UK) after prior antigen retrieval procedure (three consecutive 5-min cycles in a microwave oven set at high power, placing slides in sodium citrate buffer). Positive controls were represented by macrophages and endothelial cells, whereas negative controls were obtained by omitting the primary antibody.

Statistics

Galectin-3 immunostaining was blindly evaluated by two independent observers, with no knowledge of the histological diagnosis, using the following semiquantitative scale: - = no reactivity or some neoplastic cells with nuclear reactivity; + = more than 10% of the neoplastic cells with positivity in the cytoplasm or in both the cytoplasm and the nucleus.
Sensitivity, specificity, positive/negative predictive values, and diagnostic accuracy of galectin-3 test in presurgical cytological samples were then separately assessed for both overall carcinomas vs adenomas and MICs vs adenomas. The final histological diagnosis was accepted as the gold standard.

RESULTS

All the papillary carcinomas showed a strong cytoplasmic and nuclear galectin-3 immunostaining in both cell-blocks (Fig. 1A) and their corresponding histological counterparts. Thirty-four out of 39 follicular carcinomas stained positively in cell-blocks (Fig. 1B), whereas all their histological counterparts showed galectin-3 immunoreactivity in the cytoplasm or in both the cytoplasm and the nucleus. Among adenomas, 93 samples out of 105 were galectin-3 negative in both cell-blocks and surgical samples (Fig. 1C), whereas 12 cases showed galectin-3 immunoreactivity in both FNAB and surgical materials. Five positive adenomas had a histological diagnosis of microfollicular adenoma with lymphocytic thyroiditis, and 7 of Hürthle-cell adenoma with marked cellular atypia. In 3 of the latter ones, a piecemeal infiltration of the inner border of the capsule, not satisfying currently accepted histomorphological criteria of malignancy, was found (Fig. 1D). Histiocytes and endothelial cells were galectin-3 positive, whereas normal follicular thyroid cells were negative. Galectin-3 test sensitivity and specificity in all carcinomas vs adenomas were 92.3% and 88.6%, respectively. Positive and negative predictive values were 83.3% and 94.9%, respectively. Galectin-3 diagnostic accuracy was 90.0% (Table 1).
As far as MICs are concerned, 20 cases out of 22

Table 1 - *Galectin-3 expression in cytological cell-block specimens compared with the corresponding histological diagnoses.*

	Histology (no.=170)		
	Benign lesions	Carcinomas (no. = 65)	
Galectin-3 (no.=170)	Follicular adenoma (no.=105)	Papillary (no.=26)	Follicular (no.=39)
Positive (no.=72)*	12	26	34
Negative (no.=98)	93	0	5

*: Cytoplasmic and/or cytoplasmic and nuclear galectin-3 immunoreactivity.

Table 2 - *Galectin-3 expression in minimally invasive carcinoma cell-block specimens compared with the corresponding histological diagnoses.*

	Histology (no.= 127)	
Galectin-3 (no.=127)	Follicular adenoma (no.=105)	MIC (no.=22)
Positive (no.=32)*	12	20
Negative (no.=95)	93	2

*: Cytoplasmic galectin-3 immunoreactivity; MIC: minimally invasive carcinoma

were galectin-3 positive in both cytological samples and histological counterparts. Only two trabecular carcinomas showed galectin-3 immunostaining in the histological specimens, but not in the corresponding FNAB cell-blocks. Sensitivity and specificity of galectin-3 method in MICs vs adenomas were 90.4% and 88.6%, respectively, whereas its positive and negative predictive values were 62.5% and 97.9%, respectively. Galectin-3 diagnostic accuracy in presurgically detected MICs was 88.9% (Table 2).

DISCUSSION

In this study, galectin-3 expression was re-assessed in a large collection of cytological and histological paraffin-embedded thyroid samples using a commercial monoclonal antibody against human galectin-3 and a biotin-free detection system in order to avoid possible interfering technical factors, which can lead to some of the discrepancies reported in literature data (36-38). Indeed, since thyroid neoplasms show endogenous biotin-like activity especially when heating-antigen retrieval methods are applied (40, 41) the employment of a biotin-streptavidin-based staining can lead to false positive results.

The results of this study further confirm the selective expression of galectin-3 in malignant follicular thyroid tumors, but not in benign and normal thyrocytes, thus corroborating our previous data that had been obtained with a biotin-based immunostaining and/or a non-species-specific monoclonal antibody. In particular, the data reported here support the notion that only cytoplasmic galectin-3 expression has to be considered as a specific sign of thyroid malignancy, regardless of nuclear immunostaining (39). In fact, nuclear expression may represent an index of thyroid cell proliferation rather than malignant transformation, since nuclear galectin-3 immunoreactivity was found in both adenoma cells and normal thyrocytes. This observation agrees with the results of several studies showing the physiological involvement of galectin-3 in nuclear mRNA splicing of non-transformed cells (28).

Recently, some papers have reached different conclusions on the predictive value of galectin-3 expression in thyroid neoplasms, suggesting that its expression may not be selective for malignant lesions (42-44). These reported discrepancies could be attributable to the RT-PCR-based technique used by the authors to assess galectin-3 expression. In fact, the RT-PCR inability to discriminate the galectin-3 cytoplasmic rate from the nuclear one makes it unsuitable for diagnostic purposes, as only the cytoplasmic galectin-3 expression is suggestive of thyroid malignancy. Likewise, this technique is not able to distinguish the galectin-3 mRNA rate deriving from endothelial cells, histiocytes and fibroblasts, that are usually present in variable amounts in thyroid tissue, from the mRNA specifically present in thyrocytes, thus providing a large number of false positive results (39).

In this study, cytoplasmic galectin-3 immunoreactivity was detected in all samples of papillary carcinomas, including their follicular variants, thus highlighting the high sensitivity of the immunohistochemical test in identifying these lesions. Although the presurgical cytological diagnosis of papillary carcinoma is usually easy, it may encounter some diagnostic difficulties in

the detection of both the follicular variants, and certain poorly-differentiated types, that often result in an indeterminate FNAB cytological diagnosis (in our series, 6 follicular variants and 1 tall-cell type were misdiagnosed at cytological assessment). Therefore, galectin-3 proves to be a sensitive and reliable marker, able to reduce the number of inconclusive presurgical diagnoses of thyroid papillary neoplasms.

As far as follicular carcinomas are concerned, galectin-3 showed a high sensitivity and specificity in follicular cancer detection on histological samples, but failed to identify 5 out of 39 cytological cases on cell-block material (5 poorly-differentiated cases: 4 trabecular, and 1 insular type). In our opinion, these immunoreactivity discrepancies between cytological and histological samples may be ascribed either to a technical error occurring in immunocytochemical critical steps (e.g. antigen retrieval process), or to a FNAB failure in providing galectin-3-representative cellular elements due to sampling in negative areas of the nodule, that have been described as frequently present within poorly-differentiated malignant lesions (34). Hence, galectin-3 test showed 12.8% of false-negative cytological results, but it has to be considered that all the false-negative cases were cytologically defined as "follicular neoplasm", for which the conventional cytology lacks in diagnosis. This worrisome percentage of false-negative galectin-3 tests in "follicular neoplasms" would be significantly reduced if galectin-3 results were integrated with the clinical findings. In fact, 3 out of 5 of our galectin-3 false-negative cytological cases (3 widely invasive follicular carcinomas) showed features strongly suspected of malignancy already at clinical evaluations, thus reducing the percentage of false-negativities to 5.1% when only the false-negative cases at both clinical and immunocytochemical examination are considered (2 MICs/39 follicular carcinomas). In our opinion, patients with an indeterminate FNAB result and a negative galectin-3 test without clinical signs suggestive of malignancy should be carefully followed up with repeated FNAB and galectin-3-test, and referred to the surgeon when an increase in the nodule size or galectin-3 positive result is noted. At worst, the diagnosis of low-grade thyroid malignancy will be just postponed.

Among follicular adenomas, we observed 5 positive cases in which lymphocytic thyroiditis encompassed neoplastic nodules. Usually, morphological finding of clusters of galectin-3 positive thyrocytes intermingled with activated lymphoid cells can be detected both in normal thyroid tissue and follicular adenomas (39). Thus, we could suppose a lymphocytic influence on neoplastic cell gene expression (including galectin-3) by paracrine secretion of hormonal cues (e.g. cytokines), so that these cases may be considered as real false positive lesions. When galectin-3 positive follicular adenomas with atypia are considered, the concordance of galectin-3 cytological and histological immunoreactivity of the samples prompts us to hypothesize that they might be real follicular carcinomas, in which capsular and/or vascular invasion was not detected or, alternatively, not apparent yet. Although the progression of follicular adenoma vs carcinoma remains an unsolved dilemma in thyroid tumorigenesis, nowadays this hypothesis finds further and stronger support in the uninterrupted flow of data provided by *in vitro* and *in vivo* molecular genetic experiments on thyroid genetic abnormalities peculiarly related to the neoplastic phenotypes (45-48). The question is quite relevant, since it would make it necessary to include both the molecular and the morphological features in the differential diagnostic criteria of thyroid malignancy. In our opinion, all patients with a FNAB cytology of "follicular neoplasm" and a positive galectin-3-cytological test should undergo surgical excision.

In conclusion, galectin-3 immunodetection is an effective diagnostic method for thyroid carcinoma of follicular origin. Since it is burdened by some false-positive/-negative rates, we recommend caution in the use of galectin-3 method free from the clinical and cytological context. Only the integration of galectin-3 immunocytochemical evaluation with both clinical and classical cytomorphological findings represents a reliable approach to the preoperative characterisation of thyroid neoplasms. Galectin-3 proves also to be a sensitive marker for MIC identification, partially solving the diagnostic dilemma represented by these tumors. In addition, it could be useful for a better histomorphological definition of MICs, alerting the pathologist toward a deeper search for minimal vascular or capsular invasions by examining a higher number of tissue sections. The routine correct use of this molecular test, by increasing the diagnostic accuracy of conventional cytology, improves the management of thyroid nodules cytologically defined as "follicular neoplasms", and leads to a reduction of useless thyroid surgical operations.

Finally, a particular consideration has to be made on the advantages of galectin-3 assessment in cell-block material, an easy and cheap technical procedure adopted only by a small minority of thyroid cytopathologists. Cell-block preparation increases cellular yield by capturing any small thyroid tissue fragments or scattered thyrocytes in the fluid specimens obtained by FNAB, meanwhile removing red blood cells. In addition, it allows the cutting of thin serial sections that can be indefinitely kept at room temperature, and provides a better morphology

than that supplied by direct cytology on smears, which represents the standard procedure for most pathologists. Hence, we suggest following the cell-block procedure and using a biotin-free immunodetection system in order to enhance galectin-3 test.

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