Galectin-3-expression analysis in the surgical selection of follicular thyroid nodules with indeterminate fine-needle aspiration cytology: a prospective multicentre study

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Summary

Background In the USA, about 30 200 well-differentiated thyroid carcinomas were diagnosed in 2007, but the prevalence of thyroid nodules is much higher (about 5% of the adult population). Unfortunately, the preoperative characterisation of follicular thyroid nodules is still a challenge, and many benign lesions, which remain indeterminate after fine-needle aspiration (FNA) cytology are referred to surgery. About 85% of these thyroid nodules are classified as benign at final histology. We aimed to assess the diagnostic effect of galectin-3 expression analysis in distinguishing preoperatively benign from malignant follicular thyroid nodules when FNA findings were indeterminate.

Methods 544 patients were enrolled between June 1, 2003, and Aug 30, 2006. We used a purified monoclonal antibody to galectin-3, a biotin-free immunocytohistochemical assay, and a morphological and phenotypic analysis of FNA-derived cell-block preparations. Galectin-3 expression analysis was applied preoperatively on 465 follicular thyroid proliferations that were candidates for surgery, and its diagnostic accuracy was compared with the final histology.

Findings 31 patients were excluded because they had small galectin-3-negative thyroid nodules; we did not have data for 47 patients; and one patient with an oncocytic nodule was excluded. 331 (71%) of the assessable 465 preoperative thyroid FNA samples did not express galectin-3. 280 (85%) of these galectin-3-negative lesions were classified as benign at final histology. Galectin-3 expression was detected, instead, in 134 of 465 (29%) thyroid proliferations, 101 (75%) of which were confirmed as malignant. The overall sensitivity of the galectin-3 test was 78% (95% CI 74–82) and specificity was 93% (90–95). Estimated positive predictive value was 82% (79–86) and negative predictive value was 91% (88–93). 381 (88%) of 432 patients with follicular thyroid nodules who were referred for thyroidectomy were benign at final histology. Galectin-3 expression was detected, instead, in 134 of 465 (29%) thyroid proliferations, 280 (85%) of these galectin-3-negative lesions were classifi ed as benign at final histology.

Interpretation Our findings show that if the option of surgery was based theoretically on galectin-3 expression alone, only 134 thyroid operations would have been done in 465 patients; therefore a large proportion (71%) of unnecessary thyroid surgical procedures could be avoided, although a number of galectin-3-negative cancers could be potentially missed. The galectin-3 test proposed here does not replace conventional FNA cytology, but represents a complementary diagnostic method for those follicular nodules that remain indeterminate.

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Introduction

Thyroid nodules are a common clinical problem. The prevalence of palpable thyroid proliferations in adults increases with age (mean prevalence 4–7% in the USA), but is much higher when subclinical nodules are also counted. About 80–80% of these lesions are benign, therefore a reliable and systematic approach to their characterisation is needed.

Thyroid fine-needle aspiration (FNA) has substantially improved the characterisation of thyroid nodules and has led to decreases in health-care costs and improved diagnosis of malignant lesions when thyroidectomy is done. However, FNA has intrinsic limitations in distinguishing between benign (nodular hyperplasia, follicular adenoma and its variants) and malignant follicular lesions (follicular thyroid carcinoma, oncocytic carcinoma, and follicular variant of papillary carcinoma). FNA fails to distinguish between benign and malignant disease in about 15–30% of tests, depending on the diagnostic centre. Consequently, many patients with follicular proliferations are referred for thyroideectomy without real therapeutic necessity: Furthermore, final histology often confirms malignancy in about 10–15% of excised lesions.

To improve the diagnostic accuracy of thyroid FNA cytology, we proposed a test method (galectin-3 thyrotest) based on expression analysis of galectin-3 on FNA-derived cell blocks. Galectin-3 is a β-galactosyl-binding molecule in the lectin group, and is involved in different biological functions, including cell adhesion, cell-cycle regulation, apoptosis, and tumour progression. We used the
galectin-3 test method for the preoperative characterisation of thyroid nodules for several reasons: first, in normal conditions galectin-3 is not expressed in the cytoplasm of thyroid cells and its forced expression (by galectin-3 cDNA transfection) generates a transformed phenotype;24-26 conversely, inhibition of galectin-3 expression has been shown to revert the transformed phenotype in different tumour models.27-29 Second, the aberrant expression of galectin-3 blocks the apoptotic programme, a feature that favours the development of cancer.30,31 Third, we had previously shown19 that galectin-3 is a physiological target of P53 transcriptional activity, and that P53-mediated down-regulation of galectin-3 is needed for P53-induced apoptosis. Fourth, published studies showed that well-differentiated thyroid carcinomas almost always express galectin-3, whereas healthy thyroid tissue and most benign thyroid proliferations do not.32,33

The main reason that conventional FNA cytology fails to characterise reliably follicular thyroid nodules in a third of tests is because cytological criteria for distinguishing benign from malignant follicular proliferations do not exist, despite progress in FNA cytology.1,3-7,20 To assess the diagnostic accuracy of the galectin-3 test method we aimed to undertake a national, prospective, multicentre study of 465 patients in collaboration with 11 specialised thyroid institutions. The final histological diagnosis (considered as the gold standard) was compared with the preoperative diagnostic findings of the test method.

**Methods**

**Patients and procedures**

Candidate working groups were selected on the basis of their recorded clinical activity (eg, number of fine-needle aspirations of the thyroid (FNAB) each year; number of thyroid ultrasonographies each year; number of aspirations of the thyroid (FNAB) each year; number of FNA preparations was done by two expert and independent pathologists at our institutions (preoperative diagnoses), and at least two experienced pathologists at final histological assessment of the 465 excised thyroid nodules measuring less than 1 cm in diameter. The final histological assessment of the 465 excised thyroid lesions was done by at least two experienced pathologists in each specialised thyroid centre (no substantial internal diagnostic disagreement was reported).

A blind central review of 294 unselected histological preparations was done by two expert and independent pathologists (Juan Rosai, Centro Diagnostico Italiano, Milan, Italy, and Virginia LiVolsi, Department of Pathology, University of Pennsylvania, PA, USA) for quality control and an “other” group of institutions in northern and southern Italy; webfigure). Operative protocols for patient selection, ultrasonography-guided thyroid FNA, cell-block preparation, and immunohistocytchemistry were provided in advance to all participating centres during two workshops. Patients were enrolled into the study between June 1, 2003, and Aug 30, 2006. All patients had a follicular thyroid nodule cytologically classified as Thy3.35 465 of 544 patients enrolled in this study were referred to surgery based on clinical reasons and multidisciplinary assessment. For the other 79 patients who were initially enrolled: 31 were not considered for surgery because they had small galectin-3-negative thyroid nodules (ie, nodules <1 cm in size or suspected colloid nodules that were present for several years without evidence of modifications), which were deemed benign after a clinicopathological consensus meeting. These patients are in active follow-up without signs of thyroid disease. We do not have information on 47 patients who received FNA cytology and galectin-3 assessment in our institutions (preoperative diagnoses), but were treated elsewhere in regional hospitals. One patient with an oncocytic nodule was removed from the study after the central blind revision of histological diagnosis because of inadequate sampling, where the sample was deemed necrotic and undefined.

70 (15%) of 465 thyroid proliferations showed atypical cells at FNA cytology, whereas 395 (85%) of 465 did not show cytological atypia. 98 (21%) of 456 follicular thyroid nodules showed oncocytic features (ie, presence of oxyphilic cells).

The study group included 355 (76%) women and 110 (24%) men, mean age 50 years (range 21-76). Mean size of the single or dominant nodule object of study was 2.8 cm (range 1.0–5.5). To obtain adequate FNA cytology and cell-block preparations, and to increase the possibility of surgical removal of the lesion, participating centres were discouraged in the workshops from enrolling patients with thyroid nodules measuring less than 1 cm in diameter. The final histological assessment of the 465 excised thyroid lesions was done by at least two experienced pathologists in each specialised thyroid centre (no substantial internal diagnostic disagreement was reported).

A blind central review of 294 unselected histological preparations was done by two expert and independent pathologists (Juan Rosai, Centro Diagnostico Italiano, Milan, Italy, and Virginia LiVolsi, Department of Pathology, University of Pennsylvania, PA, USA) for quality control of the gold standard (final histology). The lesions included in this study were: thyroid carcinomas (follicular types and follicular variants of papillary carcinoma) in 130 (28%) of 465 patients and benign thyroid proliferations (adenomas and hyperplasias) in 302 (65%) of 465 patients. 33 (7%) of 465 lesions were histologically classified as follicular tumours of uncertain malignant potential (FT-UMP).21-25 (table 1). The proportions of thyroid lesions are those expected according to national trends, with the exception of FT-UMP. This specific cluster of lesions was initially
preferentially diagnosed in central Italy, but after the blind central review (of the internal histological diagnosis), we noted that such lesions were well distributed across the participating centres in all regions. The aim of this prospective study was to assess the diagnostic accuracy of galectin-3 expression analysis on the preoperative characterisation of 465 patients with follicular thyroid nodules that FNA cytology could not distinguish as benign or malignant, and who were candidates for partial or total thyroidectomy. These thyroid proliferations were classified as Thy3 according to guidelines of the British Thyroid Association, Royal College of Physicians, London, UK. This study was done according to the ethical guidelines of the Declaration of Helsinki. Written informed consent was obtained from each enrolled patient. Specific approval was also obtained from each Institutional Scientific Board.

Galectin-3 expression analysis and immunocytohistochemistry
Operative protocols and homogeneous reagents for immunostaining were provided in advance to all centres. Immunocytohistochemistry was done as described elsewhere. Briefly, antigen-retrieval microwave treatment (0.01 M citrate buffer, pH 6.0) was applied for three cycles of 5 min each at 750 W. Endogenous peroxidase activity was quenched with methanol-hydrogen peroxide (3%) for 15 min. After blocking with unrelated antiserum, slides were incubated with the primary rat monoclonal antibody to galectin-3 (Mabtech, Nacka, Sweden) at a concentration of 10 μg/mL in a moist chamber at 4°C. The use of formalin-fixed and paraffin-embedded tissues as target preparations as positive if more than 5% of thyroid cells showed an evident cytoplasm immunostaining. Thyroid foam macrophages, often detected in histological and cytological thyroid preparations, express galectin-3 constitutively, and were deemed internal positive controls. Immunocytohistochemistry and final histology were assessed by at least two pathologists in each centre. All pathologists and cytologists participating in this study were asked to follow strictly the diagnostic criteria for definition of follicular thyroid proliferation reported in the current published studies. More specifically, adequate FNA-derived cell blocks were defined as containing at least four or five groups of follicular arranged thyroid cells with 10–20 cells in each group.

Follicular carcinoma was histologically diagnosed when capsular penetration or vascular invasion, or both, were unequivocally shown by adequate tissue sampling. Papillary thyroid carcinoma and its variants were diagnosed on the basis of nuclear features (nuclear clearing, groves, and pseudo-inclusions) and papillary or follicular architecture (or both).

As for the histological diagnosis, a blind central review of the cytological preparations was considered, but was extremely difficult for several reasons. Most importantly, there were legal issues in collecting and sending the slides for central review because they could not be duplicated. Specific authorisation from each patient and cytology services were needed, but these tasks were too time-consuming.

Website for data collection and analysis
Clinical, cytological, and pathological data, and the findings of galectin-3 expression analysis were collected for 544 patients over a period of 3.5 years. A dedicated web-based system was developed for data collection and analysis. The system consists of a website connected to a centralised database that uses PHP (Hypertext Preprocessor) and HTML (HyperText Markup Language) scripting languages. Data were stored by use of Microsoft Access and MySQL. Password-protected access to this website enabled each centre to enter patients’ data in one or more sessions by completing specific electronic forms. A JavaScript program filtered the data entered before their online submission. All data were hosted and protected on a secure server of an internet provider. At the end of the study, the entire set of data on 465 eligible thyroid proliferations was downloaded and analysed.

Statistical analysis
Sensitivity, specificity, and positive and negative predictive values of galectin-3 thyrotest were assessed as reported elsewhere. Areas under the Receiver Operating Characteristics (ROC) curves were calculated to describe the overall performance of the test by use of Stata 9.2 (version 9.0). Formal tests of intercentre variability, based on Fisher’s exact test for heterogeneity with five degrees of freedom, were done separately for sensitivity and specificity, and by analysis of two homogeneous groups of lesions: thyroid carcinomas and benign thyroid follicular proliferations. The 33 patients with FT-UMP were not included in the statistical analysis because they were indeterminate at the final histology.

Role of the funding source
The funding source had no role in study design, data collection, analysis, or interpretation, or in the writing of the report. AB had full access to all data and had final responsibility to submit the report for publication.
Results

Galectin-3 expression was absent in 331 (71%) of 465 thyroid nodules assessed preoperatively (table 2). 280 (85%) of these galectin-3-negative lesions were diagnosed as benign at final histology, whereas 29 (9%) were diagnosed as thyroid cancer. These false-negative tumours included 19 follicular variants of papillary carcinomas (FVPCs), eight follicular carcinomas (including four oncocytic variants), and two poorly differentiated carcinomas.

8 (28%) of 29 false-negative carcinomas showed variable galectin-3 expression when the test method was applied postoperatively to the corresponding excised tumours (data not shown). This observation probably suggests technical inaccuracies with FNA sampling or cell-block immunostaining, or both. However, the remaining 21 galectin-3-negative carcinomas (at preoperative and postoperative assessment) were undetectable with the proposed immuno-diagnostic approach.

Galectin-3 expression on preoperative FNA-derived cell blocks was noted instead in 134 (29%) of 465 patients (table 3). 101 (75%) of these 134 tumours were confirmed as malignant. In 22 patients, galectin-3-positive nodules were categorised as benign, whereas 11 patients had nodules that were finally classified as borderline lesions (ie, FT-UMP). 19 of the 22 patients had galectin-3-expressing benign thyroid proliferations classified as adenomas, and only three patients had nodular hyperplasia at final diagnosis.

The possibility that these galectin-3-positive follicular proliferations (19 adenomas of 176 tested and 11 FT-UMP) could represent early carcinomas, in which the histological hallmarks of malignant disease (ie, capsule and vascular invasion) is not evident yet has been previously reported elsewhere.25,26 We plan to assess this hypothesis at phenotypic and molecular levels. As expected, only three of 126 histologically confirmed hyperplastic nodules were deemed galectin-3 false-positive lesions (tables 1–3). The diagnosed thyroid malignancies were mostly follicular variants of papillary carcinoma (86 of 130 [66%] of the tumours), 37 (28%) were follicular carcinomas (15 [12%] were conventional and 22 [17%] were oncocytic variants) and seven (5%) were poorly differentiated carcinomas. The unexpected large number of follicular variants of papillary

<table>
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<th>Lesions analysed (n=465)</th>
<th>Galectin-3 positive (n=134)</th>
<th>Malignant (n=101)</th>
<th>Benign (n=220)</th>
<th>Borderline (n=11)</th>
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<td>17</td>
<td>14</td>
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*Included 19 follicular adenomas and three nodular hyperplasias.

<table>
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<th>Lesions analysed (n=465)</th>
<th>Galectin-3 negative (n=331)</th>
<th>Malignant (n=29)</th>
<th>Benign (n=280)</th>
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*8 of 29 samples showed a variable galectin-3 expression at final histology.
carcinoma highlights the fact that conventional cytology fails to correctly identify these lesions for a large number of patients.\textsuperscript{1,2,5} 381 (88%) of 432 patients with follicular thyroid nodules who were referred for thyroidectomy were correctly classified preoperatively by use of the galectin-3 test. During the central histological review of unselected thyroid lesions for quality-control purposes, the external reviewers noted 21 major discrepancies in our diagnoses of thyroid cancer, which were promptly discussed and revised independently of immunoreactivity findings. These thyroid proliferations that were not correctly classified at both preoperative cytology and at final histology probably represented a real diagnostic variable in thyroid pathology. We noted that the preoperative use of galectin-3 immunostaining could resolve about 50% of these lesions (table 4).

There were 29 false-negative lesions and 22 false-positive lesions. The overall sensitivity of the galectin-3 test was 78% (95% CI 74–82) and specificity was 93% (90–95). With a prevalence of 30% (ie, 130 of 432; 95% CI 26–34), the estimated positive predictive value was 82% (79–86) and the negative predictive value was 91% (88–93). The estimated ROC was 85 (81–88), which suggested good overall accuracy of the test, and 381 (88%) of 432 lesions were correctly classified.

The variability between centres was assessed separately for positive and negative thyroid nodules at the final histology (tables 5 and 6). In patients with cancer, positive findings varied significantly between centres (range from 39% to 94%); however, the proportions of galectin-3-positive tests in thyroid cancers was fairly consistent for those contributing centres that reported more than 30 thyroid lesions (94% and 86%). Fisher’s exact test confirmed the variability between centres (p=0.001). When the capability of each centre to correctly identify galectin-3-negative nodules was assessed, we recorded little variation (range 86–100), and Fisher’s exact test confirmed this finding (p=0.313).

Discussion

The high prevalence of thyroid nodules in the adult population (19–67% of randomly selected individuals)\textsuperscript{2,19} and the low prevalence of thyroid cancers makes the diagnosis of thyroid cancer very difficult.\textsuperscript{2,26} Our findings show that 381 (88%) of 432 patients with follicular thyroid nodules who were referred for thyroidectomy were correctly classified preoperatively by use of the galectin-3 test. Therefore, many unnecessary thyroid operations could be avoided.\textsuperscript{2,19,22,23} The overall sensitivity of the test was 78% (95% CI 74–82) and the specificity was 93% (90–95). The estimated positive predictive value was 82% (79–86) and the negative predictive value was 91% (88–93).

Notably, in 29 (22%) of 130 patients with thyroid carcinoma, the diagnostic test did not detect malignant cells preoperatively, but technical issues were probably responsible for the diagnostic failure in some of these patients. This observation is substantiated by the fact that in eight (28%) of these thyroid proliferations, which were analysed after thyroidectomy, galectin-3 expression was finally shown by immunohistochemistry (table 2, and data not shown). Furthermore, we noted that a centre contributing 20 follicular thyroid proliferations (six follicular variants of papillary thyroid carcinoma, six oncocytic adenomas, and eight follicular adenomas) provided only galectin-3-negative findings. This suggests technical failure during the galectin-3 thyrotest occurred in this centre. However, specific training and dedicated workshops on use of galectin-3 expression techniques might lower the number of galectin-3-positive cancers that are missed. Nonetheless, at least 21 (16%) of 130 thyroid carcinomas analysed here do not express galectin-3; this is probably because of additional molecular alterations that affect LGALS3 gene transcription, and we are currently studying this possibility.

As expected, we noted 19 galectin-3-positive follicular adenomas of 176 tested adenomas, and 11 galectin-3-positive follicular proliferations histologically classified as FT-UMP.\textsuperscript{22,23} We believe that surgical excision of these specific lesions might be advisable. By contrast, we confirmed the paucity of galectin-3 expression in thyroid hyperplasia (tables 2 and 3).

Although the diagnostic accuracy of the test method in some of the participating centres was not as good as in the other centres and could be improved by more workshops, the cumulative findings presented here clearly show that if the option of surgery was theoretically based on galectin-3 expression alone, only 134 thyroid operations would have been done—a decrease of 71%. This observation represents an important achievement considering that most of these lesions were benign, especially because patients undergoing thyroidectomy need substitute hormone treatment which can be stressful for them and affect their subsequent quality of life. However, of note, such a strategy would have missed 29 of 130 cancers—6% of the 465 studied. However, patients are only referred for thyroidectomy after careful pathological and clinical controls.
assessment of each specific thyroid lesion, and some suspicious, but galectin-3-negative, nodules could also be referred for surgery for clinical or cytological reasons (or both). Furthermore, the thyroid lesions analysed in this study represent a highly selected sample, from which patients with obvious cancer and obvious benign lesions have already been removed. Therefore, the 29 galectin-3-negative thyroid cancers we noted represent a group of missed lesions in a sample of thyroid nodules in the participating centres that was about five-times the size of the sample studied here. We believe that an estimated false-negative proportion of around 1.2% might be acceptable for a second-level diagnostic test such as the galectin-3 test.

Although more than 120 studies on galectin-3 expression in thyroid cancer have been published in the past decade with general agreement on its potential diagnostic usefulness in thyroid cancer, some researchers noted conflicting findings with use of galectin-3-expression test for diagnostic purposes. To our knowledge, this is the first prospective multicentre study to assess galectin-3 expression for diagnostic purposes and because diagnostic variability in the definition of benign and malignant thyroid lesions can occur, even between thyroid experts, some of the published studies might also have benefited from a central review of the histological diagnosis. Additionally, major methodological issues were noted in these reports. Furthermore, optimum galectin-3-expression analysis needs formalin-fixed and paraffin-embedded cytological preparations and a biotin-free immunohistochemical detection method. Galectin-3 expression should not be assessed on conventional cytological thyroid smears for diagnostic purposes, and only purified galectin-3-specific monoclonal antibodies should be used.

The paucity of cells obtained from thyroid FNA is an important limitation for cell-block preparation and galectin-3 immunostaining. We proposed a strategy to resolve this issue. Selected patients who are candidates for surgery, for whom FNA cytology is inadequate (Thy1) or inconclusive (Thy3) could be considered for preoperative ultrasonography-guided large-needle aspiration biopsy (LNAB), which uses 20-gauge needles. The diagnostic performance of the galectin-3 thyrest on LNAB-derived substrates is excellent. Furthermore, LNAB-derived cell-block preparations allow a comparative immunocytochemical assessment, on the same cytological slides, of different antigens associated with thyroid cancer.

Of these markers, the expression of human mesothelial antigen (HBME-1) and cytokeratin-19 (CK19), and paucity of thyroperoxidase immunoreactivity have been reported to be useful for this purpose. Although the biological rationale for the preferential expression (HBME-1 and CK19) or downregulation (thyroperoxidase) of these molecules in thyroid cancer is unclear, good overall diagnostic accuracy has been reported with the combined use of some of these markers. The quality of cell-block preparations (number of thyroid cells processed) is crucial for multiple and reliable immunostaining.

In conclusion, our findings show that the galectin-3 test can be used in the clinical setting, at least in specialised thyroid hospitals and cancer centres. Specific training and technical workshops are still necessary in some of the institutions involved in this study and other interested specialised hospitals, to optimise the accuracy of the galectin-3 test.

The galectin-3 test proposed here does not replace conventional FNA cytology, but represents a complementary diagnostic method for those follicular nodules that remain indeterminate. The correct approach for this preoperative characterisation of thyroid nodules always needs careful multidisciplinary assessment of each patient, according to published guidelines. When benign and malignant follicular lesions can be reliably distinguished preoperatively, a large proportion of unnecessary thyroid surgical procedures will then finally be avoided, thereby decreasing health-care costs.

Contributors
AB was the study coordinator. All the authors participated equally (according to their medical competences: endocrinology, cytopathology, thyroid surgery, and surgical pathology) in the clinical and diagnostic activity of characterising preoperatively the follicular thyroid proliferations noted in each thyroid centre or university hospital. This activity included the application of the galectin-3 test and its independent assessment. RB and MPM were responsible for data collection and statistical analysis.

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Conflicts of interest
AB has received a translational research grant from AIRC (Italian Association for Cancer Research), Rome, Italy. The other authors declared no conflicts of interest. The authors do not benefit financially from the galectin-3 test.

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