Combined analysis of galectin-3 and \( \textit{BRAF}^{V600E} \) improves the accuracy of fine-needle aspiration biopsy with cytological findings suspicious for papillary thyroid carcinoma

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Abstract

Ten to fifteen percent of fine-needle aspiration biopsy (FNAB) of thyroid nodules are indeterminate. Galectin-3 (Gal-3) and the oncogene \( \textit{BRAF}^{V600E} \) are markers of malignancy useful to improve FNAB accuracy. The objective of this study was to determine whether the combined analysis of Gal-3 and \( \textit{BRAF}^{V600E} \) expression in thyroid aspirates could improve the diagnosis in FNAB with suspicious cytological findings. Two hundred and sixty-one surgical thyroid tissues and one hundred and forty-four thyroid aspirates were analyzed for the presence of the two markers. In surgical specimens, Gal-3 expression was present in 27.4% benign nodules, 91.9% papillary (PTC) and 75% follicular (FTC) thyroid carcinomas. \( \textit{BRAF}^{V600E} \) was not detected in 127 benign nodules, as well as in 32 FTCs, while was found in 42.9% PTC. No correlation was found between \( \textit{BRAF} \) mutation and Gal-3 expression. Forty-seven consecutive FNAB suspicious for PTC were analyzed for the presence of the two markers. Of these nodules, 23 were benign at histology, 6 were positive for Gal-3, none displayed \( \textit{BRAF}^{V600E} \), and 17 were negative for both the markers. Twenty suspicious nodules were diagnosed as PTC and four FTCs at histology. Of these 24 carcinomas, 9 resulted positive for \( \textit{BRAF}^{V600E} \), 17 for Gal-3, and 22 for one or both the markers. The sensitivity, specificity, and accuracy for the presence of Gal-3 and/or \( \textit{BRAF}^{V600E} \) were significantly higher than those obtained for the two markers alone. Notably, the negative predictive value increased from 70.8 to 89.5%. In conclusion, the combined detection of Gal-3 and \( \textit{BRAF}^{V600E} \) improves the diagnosis in FNAB with cytological findings suspicious for PTC and finds clinical application in selected cases.

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Introduction

The current diagnostic standard for correct diagnosis of thyroid nodules is microscopic evaluation of thyroid cells obtained by fine-needle aspiration biopsy (FNAB), stained by may-grunwald–giemsa and or Papanicolaou methods (Miller et al. 1979, Jayaram 1985, Gharib & Goellner 1993). However, cytologic evaluation is not always simple, and interpreting thyroid cytology is challenging and requires expertise. Thus, the sensitivity and specificity of FNAB are variable and dependent on many factors including sample collection and preparation, and the skill of the pathologist. They are reported to range 70–98 and

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55–100% respectively (Gharib & Goellner 1993). However, these percentages do not take into account that 10–15% of FNABs are indeterminate (Gharib et al. 1984, Mazzaferri 1993).

The differential diagnosis between benign and malignant nodules is a challenge even to the most experienced pathologists in some difficult cases. In particular, the so-called ‘follicular patterned’ or ‘follicular lesions’, including adenomatous nodules, follicular adenomas, follicular thyroid carcinomas (FTCs), and the papillary thyroid carcinoma follicular variant (PTCfv), may pose a diagnostic dilemma (Mazzaferri 1993, Hegedus et al. 2003). In the latter, the nuclear cytological features typical of PTC are less evident in its follicular variant (few or absent nuclear grooves and pseudo-nuclear inclusions, mild nuclear enlargement) and in these cases, FNABs are at the most classified as suspicious for malignancy, and careful follow-up or diagnostic hemithyroidectomy is recommended. A large number of thyroidectomies are performed for diagnostic purposes. Therefore, alternative or adjunctive assays to improve preoperative assessment of thyroid nodules are much needed.

Several markers of malignancy have been investigated in the attempt to improve FNAB accuracy. Among the most promising, galectin-3 (Gal-3) has been studied in normal and pathological thyroid specimens by immunohistochemistry. Gal-3 is a β-galactosyl-binding protein involved in regulating cell–cell and cell–matrix interactions (Wang et al. 2004). Gal-3 is rarely detected in normal thyroid tissue and benign nodules by immunohistochemistry, while its expression has frequently been demonstrated in malignant thyroid tumors (Orlandi et al. 1998, Saggiorato et al. 2001, Bojunga & Zeuzem 2004). While Gal-3 sensitivity is high, there is some concern about its specificity because Gal-3 expression has been found in adenomatous goiter and Hashimoto’s thyroiditis by immunohistochemistry (Niedziela et al. 2002). Moreover, a ubiquitous expression of Gal-3 mRNA in benign and malignant thyroid tumors has been described by RT-PCR (Martins et al. 2002, Takano et al. 2003).

Although Gal-3 is easily detectable on tissue specimens, some methodological problems inherent to immunohistochemical processing on FNAB specimens can generate discrepant results (Martins et al. 2002, Niedziela et al. 2002, Mehrotra et al. 2004). However, more recent studies indicate that the search for Gal-3 protein expression in combination with other markers by immunohistochemical methods may be useful to improve the diagnostic accuracy of FNAB. Immunocytochemical expression of Gal-3, HBME-1, cytokeratin-19, RET, CITED-1, and fibronectin-1 demonstrated that a combination of two or a panel of markers may more effectively distinguish benign from malignant thyroid nodules (Prasad et al. 2005, Saggiorato et al. 2005, Barroeta et al. 2006, Nakamura et al. 2006, Rossi et al. 2006).

More recently, the identification of oncogenes with pathogenetic role in the mechanism of thyroid cell transformation has provided new molecular markers of PTC. The thymine-to-adenine transversion at nucleotide position 1799 of BRAF, which results in a valine-to-glutamate substitution at residue 600 (BRAFV600E), is the most common genetic event in PTC (Kimura et al. 2003, Trovisco et al. 2004, Xing et al. 2005). Since this BRAF mutation is highly specific and offers good sensitivity as molecular marker of PTC, its detection on FNAB specimens has been proposed as a diagnostic adjunctive tool in evaluation of thyroid nodules with indeterminate cytologic findings (Cohen et al. 2003, Xing et al. 2004, Sapio et al. 2007). To date, a correlation between BRAFV600E and Gal-3 expression in PTC has not been investigated. In order to investigate whether the combined analysis of these two markers might improve the accuracy of FNAB, we compared the expression of Gal-3 in BRAFV600E positive and negative PTCs and determined the value of combined analysis of these two markers on FNAB specimens as diagnostic adjunctive tool in evaluation of thyroid nodules with suspicious cytological findings.

Patients, materials and methods

Tissue samples

Two hundred sixty-one paraffin-embedded blocks (145 benign nodules, 84 PTCs, and 32 FTCs) were retrieved from the files of the Azienda Ospedaliera Universitaria ‘Federico II’, Naples and the Ospedale ‘Umberto I’, ASO Ordine Mauriziano di Torino, Turin were entered the study after patient’s consent. All tissues had been routinely fixed overnight in buffered formalin. Standard criteria were employed to classify tumors and their variants (Rosai et al. 1992). Fifty-five were assigned to the PTC classic form (PTCcf) and nineteen to the PTCfv. All benign and malignant neoplastic samples were carefully micro-dissected under a microscope to exclude surrounding normal tissue. Study approval was obtained from the institutional review board.

Patients and FNAB

A total of 144 patients from the Azienda Ospedaliera Universitaria ‘Federico II’, and the Ospedale
Mauriziano, were entered in the study after giving their consent and with approval from the institutional review boards. Cytology samples of thyroid nodules were obtained using a 25-gauge needle passed three to four times. In both institutions, smears were classified according to Baloch & LiVolsi (2004). Special care was taken to search for mild nuclear changes, such as enlargement and folds in the absence of pseudo-inclusion. Such features of equivocal malignant atypia lead a diagnosis of suspect for PTC, whereas aspirates having microfollicular patterns with or without Hürthle cell change and scant colloid, but lacking nuclear atypia were classified as indeterminate. Inadequate aspirates had limited cellularity or poor preservation and fixation (or a combination of these factors). A specimen was qualified as satisfactory if there were six groups of epithelial cells with at least ten cells per group. Patients, whose FNAB results did not yield a clear cytological diagnosis, were subjected to a second FNA procedure, performed twice consecutively in the same session after 3 weeks according to Baloch et al. (2003). One sample was used for cytological examination and the other was dispersed into Tri-reagent buffer (Sigma) and stored at –20 °C until DNA extraction. Only 47 consecutive patients subjected to thyroidectomy after FNAB suspicious for PTC, and 47 patients with indeterminate FNAB entered the study. Fifty benign nodules were also included as controls.

DNA extraction and detection of BRAF T1799A

For nucleic acid extraction from paraffin-embedded tissues, 5 mm sections were immersed in xylene for 30 min to remove paraffin, and washed in absolute and tap water, then in 70% ethanol. The samples were subjected to digestion with 0.5% SDS and 0.5 mg/ml proteinase K at 37 °C overnight, extracted with phenol, and precipitated with ethanol in the presence of sodium acetate. DNA extraction from Tri-reagent buffer was performed according to manufacturer’s recommendations. The final pellet was resuspended in 10 μl diethylpyrocarbonate water. DNA concentration was quantitated by A260 absorbance with a BioPhotometer (Eppendorf, Hamburg, Germany). Genomic DNA (50–100 ng/sample) was used as a template. Searching for BRAF mutation was performed by mutant allele-specific PCR amplification (MASA) as previously described (Sapio et al. 2006, 2007). Two different forward primers with substitution of a single base at the end of the primer (5'-GTGATTTTGGTCTACAGT-3' and 5'-GTGATTTTGGTCTAGCTACAGA-3') were used to amplify the wild-type allele or the BRAF T1799A transversion mutation with a unique reverse primer (5'-GGCCAAAATTTAATCAGTGGA-3'). PCRs were performed with 50–100 ng genomic DNA, 0.5 μM of each primer and 2.5 U Euro-Taq DNA polymerase (EuroClone, Celnio, Italy). All primers were obtained from Primm (Milan, Italy). All PCRs were performed separately in a PTC 100 Peltier Thermal Cycler (MJ Research Bio-Rad), including an initial denaturation for 2 min at 94 °C and subsequent denaturation for 30 s at 94 °C, annealing for 30 s at 58 °C and extension for 30 s at 72 °C. All samples were re-examined for BRAF mutation at least twice. cDNAs from NPA cells (harboring BRAF T1799A transversion mutation) and from WRO cells were used as positive and negative controls respectively. Direct sequencing of a subset of positive and negative samples was also performed as a control.

Detection of Gal-3 in surgical and in cytological specimens

Gal-3 expression was evaluated by immunocytochemistry on both cytological (cell blocks) and surgical specimens. As far as cytological samples are concerned, aspirated material and the derived small tissue fragments were fixed in formalin and processed to obtain paraffin-embedded cell blocks (Saggiorato et al. 2001). Cell blocks and histological sections underwent the same immunostaining method, which was based on a biotin-free detection system (Vector Laboratories Inc., Burlingame, CA, USA). Briefly, xylene de-waxed and ethanol rehydrated sections underwent heat-induced antigen retrieval procedure in 0.01 M sodium citrate buffer (pH 6.0) by 3 min pressure cooking. Endogenous peroxidase activity was quenched with methanol–hydrogen peroxide (3%) for 15 min at room temperature. Cell blocks and tissue sections were then incubated in the blocking solution (Ab-diluting buffer; Dako Cytomation, Carpinteria, CA, USA) and subsequently with a purified rat monoclonal antibody (mAb) to Gal-3 (Mabtech, Nacka, Sweden). After a prolonged wash in PBS, the sections were incubated with a horseradish peroxidase-conjugated rabbit anti-rat immunoglobulin as secondary antisera (Mabtech). The mAb to Gal-3 was used at a concentration range of 5–10 mg/l. The immunoperoxidase enzymatic activity was visualized using 3,3'-diaminobenzidine tetrahydrochloride for 10 min. Slides were subsequently rinsed in tap water, counterstained with hematoxylin, and mounted in Entellan (Merck). Histiocytes were adopted as internal positive controls. Cases were classified as positive when more than 10% of cells were stained. Special care was taken to evaluate cytoplasmic expression only.
Statistical analysis

Analysis of the results was computed as follows: sensitivity, true-positive/(true-positive + false-negative); specificity, true-negative/(true-negative + false-positive); accuracy, (true-positive + true-negative)/all samples examined; positive predictive value, true-positive/(true-positive + false-positive); and negative predictive value, true-negative/(true-negative + false-negative).

Results were analyzed by means of the χ² of independence test with Prism (Version 3.00 for Windows; GraphPad Software, San Diego, CA, USA). The level of significance was set at ≤ 0.05.

Results

Tissue sections from paraffin-embedded archival thyroid tissues were analyzed by immunohistochemistry and by MASA for the presence of Gal-3 and BRAFV600E respectively, and their individual- and co-expression were determined (Table 1).

Detection of Gal-3 in tissue specimens

Gal-3 protein expression was determined by immunohistochemistry in 62 benign nodules (51 nodular hyperplasia and 11 follicular adenomas), in 74 PTCs (55 PTCcf and 19 PTCfv), and in 32 FTCs (Table 1). Follicular cells of benign nodules frequently expressed Gal-3 (27.4%), mostly hyperplastic nodules. The protein expression was usually present in most neoplastic cells with a clear cytoplasmic signal admixed with positive histiocytes and inflammatory cells. Among the cases scored as positive, only two samples displayed a heterogeneous staining pattern with <20% of stained cells. The majority of carcinomas expressed Gal-3, confirming its sensitivity as a marker of malignancy. Its prevalence was 91.9% in PTC, 96.4% in PTCcf, and 78.9% in PTCfv. Nuclear staining, observed in most samples of both benign and malignant cases, was not considered for scoring purposes. In FTC, the prevalence of Gal-3 positivity was lower than in PTC, as only 24 over 32 samples (75%) displayed a clear positive staining.

Detection of BRAFV600E in tissue specimens

Eighty-four PTCs (65 PTCcf and 19 PTCfv) and one hundred and twenty-seven benign lesions were analyzed by MASA for the presence of BRAFV600E (Table 1). The oncogene was not detected in any of the benign lesions, as well as in the FTCs thus confirming the reliability of this method and the specificity of BRAFV600E as PTC hallmark. The oncogene was found in 46.2 and 31.6% of PTCcf and PTCfv respectively, consistent with most of the studies present in the literature.

Gal-3 and BRAFV600E co-expression in tissue specimens

We examined the correlation between the presence of BRAFV600E determined by MASA, and Gal-3 expression determined by immunochemistry in PTC and in their classic and follicular variants. Seventy-two PTCs (53 PTCcf and 19 PTCfv) were analyzed. Besides the large overlap, no correlation was found between BRAF mutation and Gal-3 expression (P ≤ 1).

In the PTCfv group, the Gal-3 positivity clustered together with the absence of BRAFV600E, however, the association was not statistically significant.

Detection of Gal-3 and BRAFV600E in FNAB specimens

Fifty FNAB of benign nodules and ninety-four consecutive FNABs with indeterminate (n = 47) or suspicious for PTC (n = 47) findings were analyzed for the presence of the two markers (Fig. 1). Neither Gal-3 staining nor BRAFV600E was found in benign nodules. Fifteen of these nodules were subjected to thyroidectomy and all contained benign histology. In the group of indeterminate FNAB, five displayed focal Gal-3 staining with a total number of positive cells lower than 50%. None was positive for BRAFV600E and none was subjected to thyroidectomy. Within 42 indeterminate FNAB with negative Gal-3 staining, one harbored BRAFV600E. Of these, 20 patients were subjected to thyroidectomy and two carcinomas were found: one FTC and one PTC in the BRAFV600E positive nodule. In the group of nodules suspicious for PTC, 23 were positive for Gal-3. Of these, four were

Table 1 Analysis of tissue specimens. Prevalence of positivity (%) of galectin-3 (Gal-3) and BRAFV600E in benign and malignant thyroid tissues

<table>
<thead>
<tr>
<th></th>
<th>Gal-3 (%)</th>
<th>BRAFV600E (%)</th>
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<tbody>
<tr>
<td>All benign lesions</td>
<td>27.4 (17/62)</td>
<td>0 (0/127)</td>
</tr>
<tr>
<td>Nodular hyperplasia</td>
<td>15 (51)</td>
<td>0 (0/98)</td>
</tr>
<tr>
<td>Follicular adenoma</td>
<td>2 (11)</td>
<td>0 (0/29)</td>
</tr>
<tr>
<td>FTC</td>
<td>75.0 (24/32)</td>
<td>0 (0/32)</td>
</tr>
<tr>
<td>FTC</td>
<td>91.9 (68/74)</td>
<td>42.9 (36/84)</td>
</tr>
<tr>
<td>PTC</td>
<td>96.4 (53/55)</td>
<td>46.2 (30/65)</td>
</tr>
<tr>
<td>PTC</td>
<td>78.9 (15/19)</td>
<td>31.6 (6/19)</td>
</tr>
</tbody>
</table>

Number of cases is in brackets. PTC, papillary thyroid carcinoma; PTCcv, PTC classic variant; PTCfv, PTC follicular variant.

*Two samples showed focal positivity.
also positive for \( \text{BRAF}^{V600E} \) and all resulted PTC at histology. Of the remaining 19 nodules, Gal-3-positive and \( \text{BRAF}^{V600E} \) negative, 11 were PTCs, 2 FTCs and 6 were benign. Within 24 Gal-3-negative nodules suspicious for PTC, five harbored the BRAF mutation and all were PTC at histology. Of the 19 nodules negative for both the markers, 2 were FTCs and 17 were benign.

The same results are presented in Table 2 as histology versus marker expression pattern. In the group of nodules suspicious for PTC, 23 resulted a benign lesion at histology, none of which displayed \( \text{BRAF}^{V600E} \), 6 (26.1%) were positive for Gal-3, and 17 (73.9%) were negative for both markers. Twenty nodules showed PTC and four FTC at histology. Of these 24 carcinomas, 9 (37.5%) resulted positive for

**Figure 1** Gal-3 and \( \text{BRAF}^{V600E} \) analysis in benign and indeterminate FNAB. n.o., not operated.

<table>
<thead>
<tr>
<th>Histology</th>
<th>( \text{BRAF}^{V600E}^+ )</th>
<th>Gal-3+</th>
<th>( \text{BRAF}^{V600E}^- )</th>
<th>Gal-3−</th>
<th>( \text{BRAF}^{V600E}^+ )</th>
<th>Gal-3+</th>
<th>( \text{BRAF}^{V600E}^- )</th>
<th>Gal-3+</th>
<th>( \text{BRAF}^{V600E}^+ )</th>
<th>Gal-3+</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign lesions</td>
<td>0 (0)</td>
<td>26.1 (6)</td>
<td>73.9 (17)</td>
<td>0 (0)</td>
<td>26.1 (6)</td>
<td>0 (0)</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcinomas(^a)</td>
<td>37.5 (9)</td>
<td>70.8 (17)</td>
<td>8.3 (2)</td>
<td>20.8 (5)</td>
<td>54.2 (13)</td>
<td>16.7 (4)</td>
<td>24</td>
<td></td>
<td></td>
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</tbody>
</table>

\(^a\)Four FTCs and 20 PTCs; n is in brackets.

#### Table 2 Analysis of cytology samples. Prevalence of single and combined positivity of galectin-3 (Gal-3) and \( \text{BRAF}^{V600E} \) in 47 fine-needle aspiration biopsies (FNABs) suspicious for malignancy
BRAF<sup>V600E</sup>, 17 (70.8%) for Gal-3, and 22 (91.7%) for one or both the markers. Twenty-two primary tumors and eighteen benign nodules matching the needle aspirate specimens were analyzed and compared with the corresponding FNAB results to determine whether the expression of the biomarkers in histology and cytology specimens were comparable. The presence of BRAF<sup>V600E</sup> in FNAB was confirmed in all corresponding tumor tissues. In only one PTC, BRAF<sup>V600E</sup> was found only in the tissue, suggesting the possibility of a higher marker sensitivity in tissues where a larger number of cells can be analyzed and wrong sampling is avoided. Benign nodules were invariably negative for the oncogene, and Gal-3 staining on tissues confirmed the FNAB findings.

Based on these data, we evaluated the value of Gal-3 and BRAF<sup>V600E</sup> alone or in combination as a marker of malignancy in FNAB suspicious for PTC. The sensitivity, specificity, and accuracy for the presence of Gal-3 alone were 70.8, 73.9, and 72.3% respectively, while for the presence of Gal-3 and/or BRAF<sup>V600E</sup> were 91.7, 73.9, and 83% respectively (Table 3). Sensitivity and accuracy were significantly higher than those obtained for Gal-3 alone. Notably, negative predictive value for combined markers improved from 70.8 to 89.5%.

**Discussion**

Although many studies have already examined the occurrence of Gal-3 expression and BRAF mutation in primary thyroid tumors, none investigated the correlation of these two markers and whether the combination analysis has a diagnostic value. Whereas the genetic profile of cultured cell models transformed by expression of recombinant BRAF<sup>V600E</sup> has been investigated, very little is known about the molecular profile of spontaneous PTC harboring BRAF<sup>V600E</sup> (Knauf et al. 2005, Melillo et al. 2005, Mesa et al. 2006). The knowledge of molecular characteristics might help to understand biological differences between PTC subtypes, help distinguish these histotypes by immunohistochemical methods, and improve the differential diagnosis with benign lesions. In this study, the expression of Gal-3 and BRAF<sup>V600E</sup> was assessed in a series of surgical and cytological thyroid samples. The association between marker protein expression and BRAF mutation was investigated, and the possible clinical applications were evaluated.

Expression of BRAF<sup>V600E</sup> in PTC has been extensively studied. Here, we confirm its high prevalences in both PTCcf and PTCfv. As already discussed in previous reports, different prevalences observed in the literature might arise at least in part from the application of methods with different sensibility (Fugazzola et al. 2004, Puxeddu et al. 2004, Sapio et al. 2006). MASA proved to be a specific and sensitive method to detect BRAF transversion mutation as demonstrated by the absence of false-positives in 127 control tissues and by direct sequencing of a subset of positive and negative samples. As expected, the sensitivity of BRAF<sup>V600E</sup> alone was poor in suspicious FNAB (37.5%), because this oncogene is not present in the totality of PTC and is always absent in FTC. Thus, the search for BRAF<sup>V600E</sup> alone had a modest impact on the diagnosis and on the final clinical decision. However, this marker is 100% specific; thus, it finds application in the surgery-oriented cases to guide the extent of surgical resection. In solitary suspicious nodules, when a conservative surgery is recommended, the finding of BRAF<sup>V600E</sup> can prevent a second operation necessary to complete the thyroidectomy.

Gal-3 has been associated with neoplastic processes in various tissues, and it is one of the most sensitive and accurate marker of thyroid cancers as it is present in the majority of PTC. In surgical samples, Gal-3 was expressed in 91.9% of PTC, thus confirming its correlation with cell transformation and its high sensibility as a tumoral marker. Both Gal-3 and BRAF<sup>V600E</sup> were frequently expressed in PTC. Although the large overlap, no significant correlation was found between BRAF mutation and Gal-3 expression, indicating that Gal-3 expression is correlated with transformation but not directly with BRAF<sup>V600E</sup>. We also induced transient expression of BRAF<sup>V600E</sup> by transfection of expression vector (kind gift of Dr J A Fagin) in the immortalized human

**Table 3 Statistical analysis of single and combined markers in fine-needle aspiration biopsy (FNAB) suspicious for malignancy**

<table>
<thead>
<tr>
<th></th>
<th>SN</th>
<th>SP</th>
<th>Accuracy</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BRAF&lt;sup&gt;V600E&lt;/sup&gt;</strong></td>
<td>37.5</td>
<td>100</td>
<td>68.1</td>
<td>100</td>
<td>60.5</td>
</tr>
<tr>
<td>Gal-3</td>
<td>70.8</td>
<td>73.9</td>
<td>72.3</td>
<td>73.9</td>
<td>70.8</td>
</tr>
<tr>
<td><strong>BRAF&lt;sup&gt;V600E&lt;/sup&gt; and Gal-3</strong></td>
<td>16.6</td>
<td>100</td>
<td>57.4</td>
<td>100</td>
<td>53.5</td>
</tr>
<tr>
<td>Gal-3 and/or <strong>BRAF&lt;sup&gt;V600E&lt;/sup&gt;</strong></td>
<td>91.7</td>
<td>73.9</td>
<td>83.0</td>
<td>78.6</td>
<td>89.5</td>
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</table>

SN, sensitivity; SP, specificity; PPV, positive predictive value; NPV, negative predictive value.
thyroid cell line TAD-2 and no increase of Gal-3 expression was observed by immunostaining (data not shown). These data suggest that \textit{BRAF}^{\text{V600E}} does not stimulate Gal-3 protein expression directly. Also, Gal-1 mRNA level in thyroid cell lines transfected by several oncogenes showed that the expression correlates with the malignant phenotype, but independently of the oncogene (Chiariotti \textit{et al.} 1992, 1994). From a clinical standpoint, the lack of association of two or more markers can be useful to improve the diagnostic accuracy of cytological evaluation.

More debated is the specificity of Gal-3. A number of studies have reported frequent Gal-3 expression in benign thyroid lesions and in several non-thyroidal cells, including fibroblasts and inflammatory cells, making it a marker with high sensitivity but lower specificity (Cvejic \textit{et al.} 1998, Martins \textit{et al.} 2002, Prasad \textit{et al.} 2005). The presence of Gal-3-positive cells has been associated with the presence of infiltrating macrophages in benign nodular goiter and Hürthle cells in adenomas (Matesa \textit{et al.} 2007). Most of the authors report very low Gal-3 positivity in adenomas, making this molecule a useful marker in distinguishing thyroid carcinomas from adenomas. However, in clinical practice, the differential diagnosis between benign and malignant nodules, besides adenoma also includes other benign lesions more frequently expressing Gal-3. Thus, the final specificity for thyroid carcinoma of this marker is lower when applied to inconclusive FNAB. This low specificity makes the clinical application of Gal-3 debated and prompted several researchers to search for other markers to be used alone or in combination. The combined analysis of Gal-3 and cytokeratin-19, fibronectin-1, or HBME-1 has previously been investigated, demonstrating its value when applied to inconclusive FNAB (Prasad \textit{et al.} 2005, Saggiorato \textit{et al.} 2005, Barroeta \textit{et al.} 2006). However, those studies also demonstrated the limits of this analysis in adenomatous hyperplasia where Gal-3 is frequently expressed. In our study, Gal-3 was present in 26.1% of benign nodules in the group of FNAB suspicious for malignancy. This high prevalence is determined by a number of inherent factors including genetic and geographical issues, sampling, assigned cutoff, and the skill of the pathologist.

The use of a low sensitive but 100% specific marker sequentially to a less specific but very sensitive non-associated marker might refine the diagnosis of thyroid nodules with suspicious FNAB. For this reason, we analyzed the diagnostic value of Gal-3 and \textit{BRAF}^{\text{V600E}} alone and in combination in such FNABs. Some inherent factors potentially limit the clinical utility of this approach and must be considered. Differential diagnosis between benign and malignant nodules, besides PTC also includes FTC, a malignant neoplasm in which Gal-3 is less frequently detected and \textit{BRAF}^{\text{V600E}} is always absent. The absence of BRAF mutations in FTC negatively impacts on the test reducing its final sensitivity and accuracy. Indeed, the group of 47 FNABs suspicious for malignancy of our study included four FTCs, and the sensitivity and accuracy of combined markers were reduced to 91.7 and 83.0% respectively, without affecting the specificity that remained that of Gal-3 alone. However, these percentages are significantly higher than those obtained for Gal-3 (70.8 and 72.3%) and \textit{BRAF}^{\text{V600E}} (37.5 and 68.1%) alone. Notably, an important effect of the combined analysis of the two markers was on the negative predictive value. While only a modest improvement of the positive predictive value was obtained using the combined markers (from 73.9 to 78.6%), the negative predictive value increased from 70.8% for Gal-3 alone to 89.5% for Gal-3 and/or \textit{BRAF}^{\text{V600E}}. Thus, the most important result of the combined analysis of the two markers is the reduction of false-negative cases.

Total thyroidectomy is the operation of choice for multinodular goiter for the majority of endocrine surgeons (Hisham \textit{et al.} 2001, Snook \textit{et al.} 2007). Thus, total thyroidectomy is recommended for a suspicious nodule in multinodular goiter. For single benign nodular goiter, the extension of surgery is a controversial issue. Conservative surgery is an alternative choice to total thyroidectomy to reduce risk for operative complications.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Proposal of management of FNAB suspicious for malignancy by means of Gal-3 staining and \textit{BRAF} genotyping.}
\end{figure}
In our series, following the diagram proposed in Fig. 2 in 17 over 23 patients, a second surgery procedure was avoided. Thus, in our opinion, patients with single nodules with FNAB suspicious for malignancy and positive Gal-3 should be referred to total thyroidectomy. A negative Gal-3 and a negative $BRAF^{V600E}$ test should encourage the clinician to limit the surgery procedure to a diagnostic thyroidectomy. In our series, 17 patients with benign nodule were saved from total thyroidectomy and only 2 required a second intervention to complete the thyroidectomy. In 5 over 23 (21.7%) cases, BRAF genotyping demonstrated to be of clinical utility to guide the surgery directly to total thyroidectomy.

In summary, Gal-3 analysis in combination with $BRAF^{V600E}$ in cytological specimens improves the accuracy of FNAB with cytological findings suspicious for PTC reducing the false-negative diagnosis and guiding the extent of thyroidectomy.

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