Use of molecular markers in samples obtained from preoperative aspiration of thyroid

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Abstract. Several experiments have been carried out in order to find molecular markers that increase the diagnose accuracy of the Fine-Needle Aspiration (FNA), especially for thyroid lesions of undetermined significance. The growing number of published experiments on one or more of the different types of markers has started to justify the need to gather the pieces of information as a way to add evidence and guide the development of future research in the area. From the search arguments and criteria previously defined, 95 articles were selected from the electronic databases PUBMED, MEDLINE, SCOPUS and LILACS. From the 36 markers submitted to analysis and identified in preoperative FNA thyroid samples, only 10 (GAL3, CK-19, HBME-1, TPO, CD44, Telomerase, DAP IV, RAS, RET and BRAF) were assessed in more than two investigations, be it either in panel or individually. The medium, minimum and maximum values of sensibility, specificity, positive predictive value, negative predictive value and diagnose accuracy were obtained from the group of investigation, as well as the limitations and advantages of the use of each marker were identified. The BRAF mutation, for its unquestionable specificity, and the GAL3, for its regularity of average results obtained here, found in several locations in the cell as well as out of the cell, suggesting multiple functions of this molecule, were observed as holders of more expressive evidence in the effort of reducing the uncertainty of the diagnose in preoperative FNA of thyroid.

Key words: Thyroid, Fine needle aspiration, Molecular marker

FINE-NEEDLE ASPIRATION (FNA) represents one of the first choices of diagnose procedure in the clinical management of nodular thyroid diseases, given both its technical simplicity and low cost [1]. The adoption of FNA in the diagnose protocol has contributed to the selection of adequate patients for the surgical resection of the lesions [2], because the procedure can accurately define from 65% to 80% of the diagnoses. However, its limitations are acknowledged, due to the fact that the material obtained may be considered as either inadequate or scarce because of some factors, among them, the little experience of the technical executor and/or the nodule characteristics. The diagnoses can also be of undetermined significance depending on the architectural pattern and on the cytological features of the lesion, which, sometimes, may lead to misunderstandings, doubts or disagreements, for the fact that it is a diagnosis that depends on interpretation frequently based on subjective and subtle criteria [3]. Lesions of undetermined significance, which do not define the existence or absence of malignant lesions, have represented from 10% to 20% of the cytopathological diagnoses in materials obtained in preoperative FNA of thyroid. Because of such a non-conclusive situation, several patients are referred to total or partial surgical removal of the thyroid, which is the procedure particularly suitable for the occurrence of malignant nodules. However, during the histopathological evaluation of the excised piece, it has been observed that, in general, more than two thirds of the lesions initially being of undetermined significance are in fact considered as benign. For some time, several authors have suggested that the use of molecular markers, or biomarkers, represent one of the alternatives to reduce the number of false positives and false negatives in diagnosing nodular thyroid. Several research groups have tried, and others have been trying to raise the quality of diagnose of...
these lesions by assessing the expression for a specific marker or for a panel of markers. Several investigations, however, have been tested in order to show the quality of one or several markers from tissue samples resulting from partial or total thyroidectomy, for the volume, quality and availability of the material are admittedly superior. However, the main dilemma lies on the preoperative phase, and all efforts must be made focusing on the available material as well as on the quantity and quality offered by the FNA. The quantity of markers submitted to analysis has been growing, having some of these markers been assessed in one single chance, but it has not been possible to obtain the expected results yet. Some other markers were and have been submitted to investigation, due to the fact that, through them, promising results have been foreseen, and new results have come up. This review of literature is a result of the perception of the need for a careful gathering of such investigations together with their results in order to acquire knowledge about the markers or combinations that have a higher number of evidences, as well as of those markers proper to use and methodologically feasible in material resulting from FNA.

Materials and Methods

This investigation is a systematic review with secondary data analysis of other investigations that have used samples of material obtained from preoperative FNA of thyroid – recovered from electronic database PUBMED, MEDLINE, SCOPUS and LILACS –, and also of the active search for the references of such articles, between 1994 and June, 2011 (Fig. 1). The inclusion criteria defined were as follows: a) the language the article was published in: English, French, Italian, Spanish or Portuguese; b) the main or secondary objective of the article: to differentiate malignant and benign thyroid lesion from the assessment of the expression of the molecular marker in material resulting from FNA; c) the markers had been submitted to assessment during the preoperative phase; d) the results expressed directly or led to the number of true positives (TP), false positives (FP), true negatives (TF) and false negatives (FN), for each antibody or individual mutation, even when a panel of markers had been analysed; and e) the histopathology of excised piece had been considered as the gold standard of diagnose.

The articles were assessed and the data that composed a systematization form previously elaborated was obtained. The articles were classified according to the volume of information offered (Table 1), following the criteria: Class A (Excellent): from 80% to 100%; Class B (Regular): from 50 to 70%; Class C (Weak): from 0 to 40%.

The results of the expression of the marker as well as of the histopathology presented in each investigation were inserted in a contingency table 2x2 and, from the table, the main quantities assessing the diagnose tests were obtained: sensibility; specificity; predictive positive value; predictive negative value and diagnose accuracy.

Results

After reading and correlation to the defined criteria, 95 articles from different electronic database were included in this study. Most of the studies (76.8%) were related to classes B and C. Only three articles had the total of elements of systematization available. The studies included were produced by research groups from 23 different countries and taken from 43 different periodicals. Most of the articles (56.8%) were from...
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RET) and HBME-1 (Hector Battifora Mesothelial Antigen-1). These markers were referred to and involved in 81% of the investigations included here.

Thirty (31.6%) out of the ninety-five articles selected included studies with panels of markers (Table 2) and 65 (68.4%) used only one marker. In the studies with markers in panel during use, GAL3, BRAF, RET and HBME-1 have excelled, respectively. In the investigations that used only one marker, there was clear predominance of GAL3 and of the BRAF mutation.

The streptavidin-biotin-peroxidase complex was the predominant method in the immunocytochemical investigations (Table 3), followed by the detection by free biotin. Regarding the mutations or gene expressions, almost all of them were analysed by polymerase chain reaction (PCR) through different techniques of detection, such as, respectively: Direct sequencing; Light Cycler - PCR; Mutant Allele-Specific Amplification (MASA); Pyrosequencing; Dual-Priming Oligonucleotide (DPO) - based multiplex and colorimetric mutector assay.

The immunocytochemical studies composed predominantly their samples in the form of smears (56.6%), followed by a cell block (39.5%), immunoblotting (1.9%) and nonspecified (1.9%). Regarding mutations or gene expression, the samples consisted of liquid based preparations (38.6%), reprocessing smear for extraction of nucleic acid (31.8%) and wash out fluid (29.5%).

Discussion

First, it seems important to consider that, although it has been acknowledged that some markers have better qualification, sensibility or specificity, for one or more histological subtypes, due to the genetic alterations in lesion [4], either the differences of the main localization of the marker in cell or the morphological characteristics of the lesion [5], the objective of this investigation was not to assess the qualification of the markers concerning one specific histological subtype, but to identify the information register considered to be relevant (elements of systematization) in experiments with molecular markers regarding preoperative FNA of thyroid, as well as how to consolidate values expressed by them through similar methodologies.

The challenges imposed during a review like this must be acknowledged, for the fact that different research protocols are applied to the same theme, lead-
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Table 2 Study distribution according to the types and quantity of markers used in panel

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Markers used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Van Hoeven et al.</td>
<td>1998</td>
<td>HBME-1/CA19-9/CD15</td>
</tr>
<tr>
<td>Maruta et al.</td>
<td>2004</td>
<td>GAL3/CD44</td>
</tr>
<tr>
<td>Bartolazzi et al.</td>
<td>2001</td>
<td>GAL3/CD44</td>
</tr>
<tr>
<td>Gasbarri et al.</td>
<td>1999</td>
<td>GAL3/CD44</td>
</tr>
<tr>
<td>Cantara et al.</td>
<td>2010</td>
<td>BRAF/RET/RAS/TRK*/PAX8**</td>
</tr>
<tr>
<td>Salvatore et al.</td>
<td>2004</td>
<td>BRAF/RET</td>
</tr>
<tr>
<td>Moses et al.</td>
<td>2010</td>
<td>BRAF/RET/RAS</td>
</tr>
<tr>
<td>Nikiforov et al.</td>
<td>2009</td>
<td>BRAF/RET/RAS/PAX8**</td>
</tr>
<tr>
<td>Musholt et al.</td>
<td>2010</td>
<td>BRAF/RET</td>
</tr>
<tr>
<td>Sapio et al. [32]</td>
<td>2007</td>
<td>GAL3/BRAF</td>
</tr>
<tr>
<td>Sapio et al.</td>
<td>2010</td>
<td>BRAF/RET</td>
</tr>
<tr>
<td>Pizzolanti et al.</td>
<td>2009</td>
<td>CK-19/P63</td>
</tr>
<tr>
<td>Dominguez et al.</td>
<td>2008</td>
<td>CK-19/CD19/CD44/CD15</td>
</tr>
<tr>
<td>Ohori et al.</td>
<td>2010</td>
<td>CK-19/CD19/CD44</td>
</tr>
<tr>
<td>Raggio et al.</td>
<td>2010</td>
<td>HBME-1/GAL3/CD19/CK-19/CD15</td>
</tr>
<tr>
<td>Bonzanini et al.</td>
<td>2011</td>
<td>HBME-1/GAL3/CD19/CK-19/CD15</td>
</tr>
<tr>
<td>Micco et al.</td>
<td>2010</td>
<td>HBME-1/GAL3/CD19/CD44/CD15</td>
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<tr>
<td>Torregrossa et al.</td>
<td>2010</td>
<td>HBME-1/GAL3/CD19/CD44/CD15</td>
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<tr>
<td>Franco et al.</td>
<td>2009</td>
<td>HBME-1/GAL3/CD19/CD44/CD15</td>
</tr>
<tr>
<td>Torres-Cabala et al.</td>
<td>2006</td>
<td>GAL1/GAL3/S100C/VDAC1</td>
</tr>
<tr>
<td>Saggiorto et al.</td>
<td>2006</td>
<td>GAL3/HBME-1/TPO/CK-19/CD19/CD15/CD44</td>
</tr>
<tr>
<td>Rossi et al.</td>
<td>2005</td>
<td>GAL3/CD19/CD44/CD15</td>
</tr>
<tr>
<td>Pineda et al.</td>
<td>2003</td>
<td>GAL3/MUC-1/DAP IV</td>
</tr>
<tr>
<td>Asioli et al.</td>
<td>2010</td>
<td>GAL3/HBME-1/EMERIN</td>
</tr>
<tr>
<td>Torconet et al.</td>
<td>2009</td>
<td>CYCLIN D1/CYCLIND3</td>
</tr>
<tr>
<td>Aratake et al.</td>
<td>2002</td>
<td>GAL3/DAP IV</td>
</tr>
<tr>
<td>Chandan et al.</td>
<td>2006</td>
<td>CD-57/GLUT-1*</td>
</tr>
<tr>
<td>Nar et al. [23]</td>
<td>2011</td>
<td>CYCLIN A/CYCLIN B</td>
</tr>
</tbody>
</table>

*There was no mutation in the samples selected. **Only one mutation present in the sample. ***In FNA samples, the marker was negative for benign and malignant lesions. ****Postoperative FNA.

ing to variations of methodological quality, making it hard to compare previous investigations. Such methodological differences generate huge discrepancies of results among studies of several markers [6].

Although this investigation did not intend to discuss operative characteristics of laboratory techniques to identify markers, the number of differences among techniques and methods used in the studies included must be highlighted. Differences from gauge needles used for puncturing, moving to the dilution or final concentration of the marker, up to the criteria of measurement of immunostaining are identified. Some examples are:

- It was observed that six out of the seven studies analysing CK-19 (Cytokeratin-19) in preoperative FNA of thyroid, have identified dilution, and all of them were different among themselves, varying from 1:40 to 1:400. This fact was reproduced in different markers;
- Studies assessing the immunostaining of the same marker, the GAL3, presented more than four criteria of assessment: strong; mild; weak or negative; nuclear and/or cytoplasmic immunostaining; < 50% versus > 50%; < 10% versus > 10%.
- What qualifies an investigation is the existence of more than one observer, who must intervene independently. About 60% of the studies do not indicate the number of cytopathologists involved in the assessment of results shown by the marker.
- It was observed a scarcity of studies (≤ 2 studies) on 26 markers (Tables 3 and 4): CA19-9 (carbohydrate antigen 19-9) and CD15[7], CXCR4(CXC chemokine receptor 4) [8], onfFN (oncofetal fibronectin) [9], HMG1 (High Mobility Group I) [10], Ki67 and Laminin [11], Lactoferrin [12], MET(hepatocyte growth factor receptor) [13], p63 [14], S100/VDAC1(voltage dependent anion channel 1)/Galectin-1 [15], PPARgamma (Peroxisome proliferator activated receptor gamma) [16],
### Table 3  
**Distribution of markers used in preoperative FNA of thyroid by immunocytochemistry and the average values of sensibility, specificity, positive predictive value, negative predictive value, diagnose accuracy obtained.**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Number of experiments</th>
<th>Average SN</th>
<th>Average SP</th>
<th>Average PV+</th>
<th>Average PV-</th>
<th>Average AC</th>
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<td>GAL3</td>
<td>27</td>
<td>79.20</td>
<td>87.26</td>
<td>84.15</td>
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<td>78.00</td>
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<td>RET</td>
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<td>81.00</td>
<td>65.50</td>
<td>69.50</td>
<td>65.50</td>
<td>73.00</td>
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<tr>
<td>CD57</td>
<td>2</td>
<td>95.50</td>
<td>89.50</td>
<td>80.00</td>
<td>96.50</td>
<td>90.00</td>
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<td>DAP IV</td>
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<td>90.00</td>
<td>83.00</td>
<td>87.00</td>
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<td>97.00</td>
<td>93.00</td>
<td>82.00</td>
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<td>95.00</td>
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<td>94.00</td>
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<td>80.00</td>
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<td>84.00</td>
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<td>100.00</td>
<td>70.00</td>
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<td>100.00</td>
<td>89.00</td>
<td>92.00</td>
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<tr>
<td>CYCLIN D1</td>
<td>1</td>
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<td>100.00</td>
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<td>75.00</td>
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<td>KS</td>
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<td>94.00</td>
<td>70.00</td>
<td>79.00</td>
</tr>
</tbody>
</table>

**SN, sensibility; SP, specificity; PV +, predictive positive value; PV -, predictive negative value; AC, accuracy.**

### Table 4  
**Distribution of markers used in preoperative FNA of thyroid by other methods for detection of marker and the average values of sensibility, specificity, positive predictive value, negative predictive value, diagnose accuracy obtained.**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Method</th>
<th>Number of experiments</th>
<th>Average SN</th>
<th>Average SP</th>
<th>Average PV+</th>
<th>Average PV-</th>
<th>Average AC</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRAF</td>
<td>Nucleic acids extraction and PCR</td>
<td>26</td>
<td>52.35</td>
<td>97.92</td>
<td>99.85</td>
<td>51.62</td>
<td>70.54</td>
</tr>
<tr>
<td>RET</td>
<td>Nucleic acids extraction and PCR</td>
<td>11</td>
<td>18.20</td>
<td>88.73</td>
<td>87.00</td>
<td>59.60</td>
<td>55.30</td>
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<td>RAS</td>
<td>Nucleic acids extraction and PCR</td>
<td>5</td>
<td>23.00</td>
<td>97.20</td>
<td>82.20</td>
<td>63.20</td>
<td>65.00</td>
</tr>
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<td>Nucleic acids extraction and PCR</td>
<td>2</td>
<td>75.00</td>
<td>96.00</td>
<td>94.00</td>
<td>83.50</td>
<td>87.50</td>
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<td>MUC-1</td>
<td>2</td>
<td>74.50</td>
<td>95.50</td>
<td>91.50</td>
<td>85.50</td>
<td>87.50</td>
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<td>GAL3</td>
<td>Nucleic acids extraction and PCR for hTERT gene expression</td>
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<td>FIBRONECTIN</td>
<td>Nucleic acids extraction and PCR</td>
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<td>TELOMERASE</td>
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<td>46.00</td>
<td>80.00</td>
<td>55.00</td>
</tr>
</tbody>
</table>

**SN, sensibility; SP, specificity; PV +, predictive positive value; PV -, predictive negative value; AC, accuracy; PCR, Polymerase chain reaction; ELISA, Enzyme-Linked Immunoabsorbent Assay; hTERT, Human Telomerase Reverse Transcriptase; TRAP, Telomere Repeat Amplification Protocol; FISH, Fluorescence in situ hybridization.**
to the proto-oncogene RAS, i.e., a huge number of malignant lesions histopathologically confirmed did not show the presence of this mutation.

GAL3 is one of most frequently investigated molecular markers for the diagnose of the thyroid cancer [34], be it in tissues [35] or in cytological material of FNA, as shown in this study. The studies published up to the present have not offered a definite answer for the use of GAL3 in clinical practice. Methodological matters are mentioned by several authors [36-39] as being responsible for the controversial results published regarding GAL3.

Despite the fact that different and possible methodological flaws have been mentioned by several authors, GAL3 has shown in this study, by immunocytochemistry, an explicit uniformity of average value for the sensibility, specificity, positive predictive value, negative predictive value and diagnose accuracy, i.e., in all of them, GAL3 has obtained value equal or superior to 80%. The result represents much more for the continuity of research with the GAL3 with a standardization of procedures [37] than for an alleged ban of its use in FNA of thyroid [36].

The BRAF mutation has presented extraordinary average values of specificity (97.9%) and positive predictive value (99.9%) resulting from the occurrence of only seven false positive results identified in three investigations [40-42], among the 2800 malignant and benign lesions used in the 26 investigations including BRAF. The different methods used in the detection of marker do not seem to be a disadvantage, because of the fact that they present similar results [43].

Finally, the BRAF mutation, for its unquestionable specificity, and the GAL3, for its regularity of average results obtained here, suggesting multiple functions of this molecule, were observed as holders of more expressive evidence in the effort of reducing the uncertainty of the diagnose in preoperative FNA of thyroid.

**Conflict of Interest**

The authors have declared no conflict of interest.

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