Mini Review

Promise and pitfalls of molecular markers of thyroid nodules

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ABSTRACT

Thyroid nodules are common in the general population with a prevalence of 5-7% The initial evaluation of thyroid nodules commonly involves thyroid function tests, an ultrasound (USG) and fine needle aspiration biopsy (FNAB). The optimal management of patients with thyroid nodules with indeterminate cytology is plagued by the lack of highly sensitive and specific diagnostic modalities In this article we attempt to review the available literature on the molecular markers which are increasingly being studied for their diagnostic utility in assessing thyroid nodules. The various molecular markers consist of gene mutations, gene re arrangements, RNA based assays and immunohistochemical markers. The molecular markers definitely would help to optimise the management of such patients.

Key words: Molecular markers, thyroid nodules, genetic mutations, gene rearrangements

Introduction

Thyroid nodules are common in the general population with a prevalence of 5% - 7% (by palpation). The estimated prevalence of clinically inapparent thyroid nodules (by ultrasound) is even higher, around 20% to 76% with similar prevalence in autopsy studies.[1]

The initial evaluation of thyroid nodules commonly involves thyroid function tests, an ultrasound (USG), and fine needle aspiration biopsy (FNAB). On FNAB, 60% – 80% of the nodules are classified as benign, and 3.5% – 5% as malignant. 10% – 20% of the FNAB samples are reported as having indeterminate cytology.[2] These require further evaluation to distinguish between follicular adenoma (FA), adenomatoid hyperplasia, follicular thyroid carcinoma (FTC), and follicular variant of papillary thyroid carcinoma (PTC), and these patients currently have to undergo (diagnostic) surgery, which will eventually detect thyroid malignancy in about 20% of these patients.[2] This means that 80% of the thyroid FNAB samples that were classified as indeterminate by cytology will undergo an unnecessary diagnostic thyroidectomy, which has its own risk of complications. Thus, there is a need to better delineate this indeterminate category.

In this article, we attempt to review the available literature on the molecular markers, which are increasingly being studied for their diagnostic utility in assessing thyroid nodules.

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Molecular Markers

The various molecular markers consist of gene mutations, gene re arrangements, RNA-based assays, and immunohistochemical markers.

A. GENETIC MUTATIONS AND GENE RE-ARRANGEMENTS

1. BRAF MUTATION

The "MAP Kinase pathway (MAPK Pathway) is an intracellular signaling pathway, which plays a fundamental role in cell functions like

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proliferation, differentiation, apoptosis, survival, and, tumorigenesis when aberrantly activated. *BRAF* is the most potent activator of the MAPK pathway. The *T1799A* point mutation, which causes the V600E amino acid change in the BRAF protein, is the most common, accounting for more than 90% of all the BRAF gene mutations. BRAF mutations are the most commonly detected abnormality in PTC, and are seen in about 45% of sporadic adult cases but less frequently (0% - 12%) in pediatric and radiation-induced tumors.[3] In addition to papillary carcinomas, this mutation is also found in poorly-differentiated and anaplastic carcinomas. It is rarely seen in the follicular carcinomas (1% - 2%) or benign thyroid nodules, and hence it seems to be a specific marker for PTC.[4] Also, it may assist prognostically, as patients with BRAF-positive PTCs have been shown to have more aggressive disease at presentation, with higher rates of extrathyroidal extension, lymph node metastasis, and tumor recurrence after initial treatment.[5]

When studied as a diagnostic marker, a meta-analysis of 18 studies on BRAF mutations showed that the rate of malignancy in BRAF-positive nodules tested by FNA was 99.8%. Importantly, 15% to 39% of these BRAF-positive FNA samples were indeterminate or non-diagnostic by cytology, demonstrating its utility in establishing a definitive diagnosis of cancer in nodules with indeterminate cytology.[6]

2. ***RAS* MUTATION**

RAS proteins are positioned at the inner surface of the plasma membrane where they transduce extracellular ligand-mediated stimuli to a cascade of cytoplasmic proteins and MAP kinases, which, in turn, influence cell growth, differentiation, and apoptosis.[7] *RAS* mutation are the second most common mutation found in thyroid cancers,[8] and the most common type of *RAS* mutations in thyroid cancer is the N-2-*ras* mutation in codon 61.[9] The frequency of *RAS* mutations ranges from 0.9% to 23.9% in FTC, 0.6% to 15.2 % in benign follicular adenomas, and these are very rare in colloid nodules (1% - 2.5%) and papillary carcinoma (0.3% to 5%).[10] These mutations, especially those of *k-ras,* are also a marker for aggressive cancer behavior, large tumor size, vascular invasion, and distant metastasis.[11]

When studied as a diagnostic marker in FNA, *RAS* mutation was found to confer a 87.5% probability of malignancy, including a 62.5% probability of a PTC (mainly follicular variant) and a 25% probability of a FTC, thus justifying the recommendation of surgery for patients with *RAS*-positive nodules.[12] However, 12.5% of benign follicular adenomas are false positive for *ras* mutation, and these may be precursors of FTC, as has been shown in some mouse models.[13]

3. **RET-PTC RE-ARRANGEMENT**

The *RET* proto-oncogene encodes a cell membrane tyrosine-kinase receptor protein. The *ret-ptc* gene represents an intra-chromosomal gene re-arrangement wherein the *RET* tyrosine-kinase (RET-TK) domain gets fused to the 5'-terminal region of a heterologous gene, producing fusion genes that give rise to a constitutively activated form.[14] *RET-PTC* re-arrangements were identified in PTCs before *RET* was recognized as the susceptibility gene for multiple endocrine neoplasia 2 (MEN2). There are now at least 15 types of *RET-PTC* re-arrangements involving *RET* and 10 different genes, the commonest being RET-PTC1 and RET-PTC 3. These have been reported in 11% to 43% of PTCs.[14] They are also known to occur in follicular adenomas, adenomatous goiter, and in Hashimoto's thyroiditis, though in such instances, the pathology is heterogeneous while in PTC, there is clonal evolution of *RET/PTC* positive cells.[15]

Carol *et al*. observed that the identification of *RET/PTC* gene re-arrangements as an adjunct to cytology in FNACs refined the diagnosis of PTC in 60% of cases that would otherwise have been considered indeterminate and in 33% of those considered insufficient for cytological diagnosis.[16]

4. **PAX8/PPARγ REARRANGEMENT**

PAX8/PPARγ re-arrangement is a result of t(2;3) (q13;p25) translocation that leads to the fusion between the PAX8 gene and the peroxisome proliferator–activated receptor (*PPARγ*) gene.

PAX8/PPARγ re-arrangement is described to be present in 26% - 40% of FTC, variably from 0% - 37% in follicular variant of PTC and much less in Hurthle cell carcinoma (1 in 30 in one study) and hyperplastic nodules.[17] FTC patients with *PPARγ* re-arrangement more frequently have vascular invasion, areas of solid/nested tumor histology, and previous non-thyroid cancers.[18] Thus, *PAX8/PPARγ* re-arrangement typically correlates with the presence of malignancy, although in prospective studies, only a few positive cases have been reported so far.

5. **USE OF A COMBINATION OF MARKERS**

Currently, no single genetic marker is specific and

sensitive enough to reliably make a diagnosis of benign versus malignant lesion, and hence there may be an advantage using a combination of these markers. Several studies address this issue.

Nikiforov *et al*., studied a of 1,056 consecutive thyroid FNA samples with indeterminate cytology for determination of *BRAF*, *RAS*, *RET/PTC*, and *PAX8-PPARγ*. They reported that in specific categories of indeterminate cytology, i.e. atypia/FLUS (follicular lesion of undetermined significance), follicular neoplasm/suspicious for a follicular neoplasm, and suspicious for malignant cells, the detection of any mutation conferred the risk of malignancy of 88%, 87%, and 95%, respectively, as against the risk of malignancy based on cytology alone was 14%, 27%, and 54%, respectively. The risk of cancer in mutation-negative nodules was 6%, 14%, and 28%, respectively. The overall cancer rate for mutation-negative status was 6%, and among these, only 2.3% were invasive and 0.5% had extra-thyroidal extension. Hence, they recommended total thyroidectomy in mutation-positive status. For mutation-negative patients, lobectomy was recommended as the first procedure.[19]

Carolina *et al*. recently reviewed and re-analyzed the literature including 16 studies with 1 mutation [e.g., *BRAF* or *RET/PTC*] and 4 studies that have analyzed combination of several mutations (*BRAF, RAS, RET/PTC,* and *PAX8/PPARγ*). More importantly, they excluded the samples with FNA cytological diagnosis of "suspicious of malignancy" since these have high chances of malignancy (50% - 70%) and might falsely inflate the performance of molecular markers. For these 4 studies, which were re-analyzed, the combined sensitivity and specificity were 63.7% and 98%, respectively. There were 5 false positives in the 4 studies and in all of them, the *ras* mutations were detected in follicular adenomas, and the postulated explanation was that it might be a precursor of malignancy on the background of Hashimoto's thyroiditis.

One such panel for analysis of combination of markers, which is commercially available, is The Asuragen miRInform™ molecular panel, which includes various mutations in BRAF, RAS and RET-PTC and PAX8-PPARG gene re-arrangements.

B. RNA-BASED MARKERS

1. MICRO RNA (miRNA)

MicroRNAs are 21 to 22 nucleotide segments of non-coding RNA that have a key role in post-transcriptional gene regulation (mainly inhibitory) through complementary binding that mediates the translation and degradation of messenger RNA.

Many studies have found up-regulation of different miRNAs in PTCs using microarray, out of which miR-221 and -222 are the most consistently up-regulated in PTCs while miR-1, -191, -486, and -451 are consistently down-regulated.[20]

Nikiforova *et al*. observed that the most up-regulated miRNAs in conventional FTCs were miR-187, -224, -155, -222, and -221, and those in oncocytic variants were miR-187, -221, -339, -183, -222, and -197. They observed that when at least one miRNA was overexpressed more than 2-fold, the sensitivity of tumor detection was 100%, specificity 94%, and accuracy 95%, whereas when 3 or more miRNAs were up-regulated, the sensitivity of tumor detection was 88%, specificity 100%, and accuracy 98%.[21]

However, no large-scale, prospective, multicenter trial investigating microRNA has yet been performed. Presently, microRNA testing of thyroid aspirates is not commercially available and is offered only through research protocols.

2. THYROID STIMULATING HORMONE RECEPTOR mRNA

Because thyroid cancer cells express functional TSH receptors (TSHR), TSHR-mRNA in peripheral blood might serve as a tissue/cancer-specific marker. TSHR-m RNA is used as a peripheral blood marker in the follow up of patients of well-differentiated thyroid malignancy.

The diagnostic value of circulating TSHR mRNA, for pre-operative detection of differentiated thyroid carcinoma (DTC) in patients with thyroid nodules, was evaluated in 258 subjects (with 51 normal subjects), by Chia et al. They demonstrated a sensitivity of 90% and specificity of 80% of TSH mRNA to predict malignancy, especially in those with indeterminate cytology on FNAC.[22]

3. GENE EXPRESSION (MICRO ARRAY) ANALYSIS

Unlike single gene mutations or re-arrangements, microarray diagnostic tests can detect hundreds of expressed genes. It involves the use of multi-gene expression classifiers that assess gene expression from mRNA isolated from needle washings during a standard FNA procedure.

Based on the previous work by Chudova *et al*. on whole genome assays, Alexander *et al*. conducted a large, prospective, multicenter validation study of a gene expression classifier using 167 genes on 265 indeterminate nodules and found 92% sensitivity with a specificity of 52% to diagnose malignancy. The negative predictive values for "atypia/FLUS,"

"follicular neoplasm or lesion suspicious for follicular neoplasm," or "suspicious cytologic findings" were 95%, 94%, and 85%, respectively.

However, the analysis revealed 7 aspirates with false negative results, some of which might be due to insufficient sampling of the nodule. They suggested that due to high negative predictive value, the GEC can be used as a rule out test to avoid unnecessary surgeries.[23] This gene classifier (Veracyte Affirma GEC) is available commercially after the above validation study.

4. **IMMUNOHISTOCHEMICAL MARKERS**

   In recent years, immunohistological markers like galectin-3, HBME-1, fibronectin-, CITED-1, and cytokeratin-19 have been investigated for their role in discrimination between benign and malignant nodules. However, they have not been adopted in routine practice, mainly because of different methods used and because these markers show prominent overlap between follicular adenoma and differentiated thyroid carcinomas.[24]

5. **OTHER MARKERS**

   There are certain other potential markers like *TRK* mutations (too low prevalence of < 5 % in PTCs), *UbcH10* and *HMGA2* and *htert*, which are awaiting further large scale studies.[25]

Cost-Effectiveness of the Molecular Markers

Henry *et al.* recently analyzed and concluded that using molecular markers for cytologically indeterminate thyroid nodules can potentially avoid almost three fourths of currently performed surgeries in patients with benign nodules. Compared with the current practice based on cytological findings alone, use of this test may result in lower overall costs and modestly improved quality of life for these patients.[26]

Conclusion

To summarize, the optimal management of patients with thyroid nodules with indeterminate cytology is plagued by the lack of highly sensitive and specific diagnostic modalities. The molecular markers as described above definitely would help to optimize the management of such patients. The management algorithm that can be proposed taking into account the sensitivity, specificity, positive and negative predictive values of the various markers available (as suggested by Carolina *et al.*[24]) is as shown in Figure 1.

However, there are certain limitations to be considered. The current use of molecular analysis is still restricted to a few specialized laboratories. There is a need for standardization of methods of DNA and RNA (including miRNA) extraction and sensitive methods of mutation analysis. Though some studies have predicted it to be cost-effective, the cost would be a prohibitive concern, especially in a resource-limited setting like that in our country. Moreover, these markers are limited by low sensitivity (around 70% in different studies), which is expected, as the panels could detect only known mutations. Hence, in order to reduce

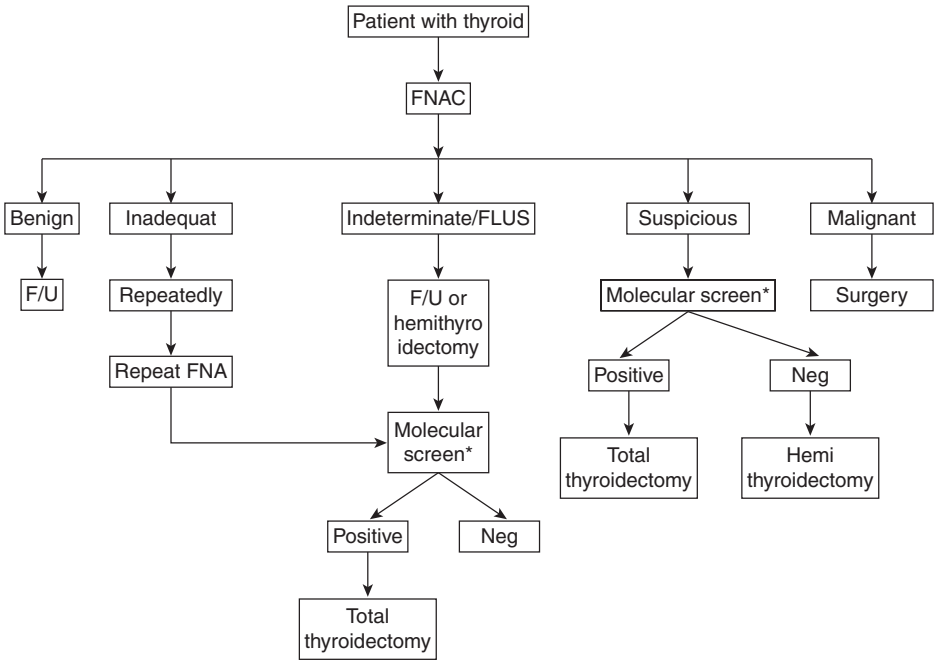

Figure 1: Algorithm for approach to thyroid nodules with molecular markers

the high number of avoidable, diagnostic thyroid surgeries, there is a need to find more markers that can reliably identify about 50% of the lesions, which are malignant but are currently mutation-negative and especially those 80% of benign nodules in the follicular proliferation/indeterminate cytology. The sampling error and inadequacy of sampling during FNAB would continue to limit the efficacy of the molecular diagnostics. Nevertheless, the scope of molecular markers in the diagnosis of thyroid nodules seem promising, especially in the cytologically indeterminate class.

References

1. Gharib H, Papini E, Paschke R, Duick DS, Valcavi R, Hegedus L, *et al.* American Association of Clinical Endocrinologists, Associazione Medici Endocrinologi, and European Thyroid Association medical guidelines for clinical practice for the diagnosis and management of thyroid nodules. Endocr Pract 2010;16(Suppl1):468-75.
2. Piana S, Frasoldati A, Ferrari M, Valcavi R, Froio E, Barbieri V, *et al.* Is a five-category reporting scheme for thyroid fine needle aspiration cytology accurate? Experience of over 18,000 FNAs reported at the same institution during 1998–2007. Cytopathology 2011;22:164-73.
3. Hassell LA, Gillies EM, Dunn ST. Cytologic and molecular diagnosis of thyroid cancers cancer. Cancer Cytopathol 2012;120:7-17.
4. Kim MI, Alexander EK. Diagnostic use of molecular markers in the evaluation of thyroid nodules. Endocr Pract 2012;18:796-802.
5. Lupi C, Giannini R, Ugolini C, Proietti A, Berti P, Minuto M, *et al.* Association of BRAF V600E mutation with poor clinicopathologic outcomes in 500 consecutive cases of papillary thyroid carcinoma. J Clin Endocrinol Metab 2007;92:4085-90.
6. Nikiforova MN, Nikiforov YE. Molecular diagnostics and predictors in thyroid cancer. Thyroid 2009;19:1351-61
7. Campbell SL, Khosravi-Far R, Rossman KL, Clark GJ, Der CJ. Increasing complexity of Ras signalling Oncogene 1998;17: 1395-413.
8. Cantara S, Capezzone M, Marchisotta S, Capuano S, Busonero G, Toti P, *et al*. Impact of proto-oncogene mutation detection in cytological specimens from thyroid nodules improves the diagnostic accuracy of cytology. J Clin Endocrinol Metab 2010;95:1365-9.
9. Xings M. Genetic alterations in the phosphatidylinositol-3 kinase/akt pathway in thyroid cancer. Thyroid 2010;20:697-706.
10. Vasko V, Ferrand M, Di Cristofaro J, Carayon P, Henry JF, de Micco C. Specific pattern of RAS oncogene mutations in follicular thyroid tumors. J Clin Endocrinol Metab 2003;88:2745-52.
11. Garcia-Rostan G, Zhao H, Camp RL, Pollan M, Herrero A, Pardo J, *et al*. Ras mutations are associated with aggressive tumor phenotypes and poor prognosis in thyroid cancer. J Clin Oncol 2003;21:3226-35.
12. Nikiforov YE, Steward DL, Robinson-Smith TM, Haugen BR, Klopper JP, Zhu Z, *et al*. Molecular testing for mutations in improving the fine-needle aspiration diagnosis of thyroid nodules. J Clin Endocrinol Metab 2009;94:2092-8.
13. Kim CS, Zhu X. Lessons from mouse models of thyroid cancer. Thyroid 2009;191317-31.
14. Tallini G, Asa SL. RET oncogene activation in papillary thyroid carcinoma. Adv Anat Pathol 2001;8:345-54.
15. Ishizaka Y, Kobayashi S, Ushijima T, Hirohashi S, Sugimura T, Nagao M. Detection of retTPC/PTC transcripts in thyroid adenomas and adenomatous goiter by an RT-PCR method. Oncogene 1991;6: 1667-72.
16. Cheung CC, Carydis B, Ezzat S, Bedard YC, Asa SL. Analysis of ret/PTC Gene rearrangements refines the fine needle aspiration diagnosis of thyroid cancer. J Clin Endocrinol Metab 2001;86:2187-90.
17. Nikiforova MN, Biddinger PW, Caudill CM, Kroll TG, Nikiforov YE. PAX8-PPARgamma rearrangement in thyroid tumors: RT-PCR and immunohistochemical analyses. Am J Surg Pathol 2002;26:1016-23.
18. French CA, Alexander EK, Cibas ES, Nose V, Laguette J, Faquin W, *et al*. Genetic and biological subgroups of low-stage follicular thyroid cancer. Am J Pathol 2033;162:1053-60.
19. Nikiforov YE, Ohori NP, Hodak SP, Carty SE, LeBeau SO, Ferris RL, *et al*. Impact of mutational testing on the diagnosis and management of patients with cytologically indeterminate thyroid nodules: A prospective analysis of 1056 FNA samples. J Clin Endocrinol Metab 2011;96:3390-7.
20. Pallante P, Visone R, Croce CM, Fusco A. Deregulation of microRNA expression in follicular cell-derived human thyroid carcinomas. Endocr Relat Cancer 2010;17:F91-104.
21. Nikiforova MN, Tseng GC, Steward D, Diorio D, Nikiforov YE. MicroRNA expression profiling of thyroid tumors: Biological significance and diagnostic utility. J Clin Endocrinol Metab 2008;93:1600-8.
22. Chia SY, Milas M, Reddy SK, Siperstein A, Skugor M, Brainard J, *et al*. Thyroid-stimulating hormone receptor messenger ribonucleic acid measurement in blood as a marker for circulating thyroid cancer cells and its role in the preoperative diagnosis of thyroid cancer. J Clin Endocrinol Metab 2007;92:468-75.
23. Alexander EK, Kennedy GC, Baloch ZW, Cibas ES, Chudova D, Diggans J, *et al*. Preoperative diagnosis of benign thyroid nodules with indeterminate cytology. N Engl J Med 2012;367:705-15.
24. Ferraz C, Eszlinger M, Paschke R. Current state and future perspective of molecular diagnosis of fine-needle aspiration biopsy of thyroid nodules. J Clin Endocrinol Metab 2011;96:2016-26.
25. Kouniavsky G, Zeiger MA. The quest for diagnostic molecular markers for thyroid nodules with indeterminate or suspicious cytology. J Surg Oncol 2012;105:438-43.
26. Li H, Robinson KA, Anton B, Saldanha IJ, Ladenson PW. Cost-effectiveness of a novel molecular test for cytologically indeterminate thyroid nodules. J Clin Endocrinol Metab 2011;96:E1719-26.

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