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Molecular Markers: From Diagnosis to Prognosis in 2013

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Abstract The detection of molecular alterations in the signaling pathways in thyroid carcinogenesis relates to some diagnostic, prognostic and treatment issues. The addition of the molecular profile to a well-differentiated thyroid cancer approach improves the accuracy of cancer detection, the prognostication and the selection of therapeutic targets for a more personalized management. It is expected that in the next few years, guidelines for thyroid cancer treatment will require a molecular marker panel to define the best treatment for this disease. BRAF and RAS mutations and RET/papillary thyroid cancer (PTC) and PAX8/PPARγ rearrangements, associated with the abnormal regulation of microRNA, constitute the most frequent molecular alterations identified in PTC. The clinical implications of the expression of these altered genes and molecules and the usefulness of these data for tailoring the approach to the disease and patient are the focus of this review.

Keywords Thyroid cancer · Molecular markers · BRAF · RET/PTC · PAX8/PPARγ · Diagnosis · Prognosis · Papillary thyroid cancer

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Introduction

Thyroid nodules can be discovered by palpation in 3–7 % of the general population, and this incidence can reach 20–76 %, depending on the patient's age, when ultrasound is added to the investigation [1]. The risk of malignancy in these nodules varies from 3.4 to 29 % and corresponds to <1 % of all human tumors, but currently the growth rate has been a high [2]. In the last 30 years, the incidence of thyroid cancer increased from 2.7 to 7.7 cases per 100,000 people in the US [3]. The discovery of a thyroid nodule leads the physician to investigate it based on some clinical and radiological criteria, which will result in a decision for observation or therapy. The main aspect to be considered in the decision-making process for a thyroid nodule investigation and treatment is the risk of malignancy [4].

Genetic and epigenetic events are considered to determine the sequence of carcinogenesis observed in thyroid cancer development. The gradual accumulation of some alterations, including somatic mutations, gene rearrangements, deletions, loss of heterozygosity, methylation, dysregulation of microRNA (miRNAs) and over- and underexpression of proteins, is found in the transformation process required for thyroid cancer development. The majority of thyroid nodules, whether benign or malignant, are diagnosed in a subclinical status and are sporadic. Some genetic alterations may be present and are associated with germline mutation syndromes such as Carney syndrome (PRKAR1A gene), Cowden disease (PTEN gene mutation), familial adenomatous polyposis/Gardner syndrome (APC gene mutation) and Werner syndrome (WRN gene mutation) [5]. It is known that 5–10 % of papillary thyroid cancers (PTC) are familial and have a multigenic component. Some mutated genes that are players in thyroid differentiation and are involved in the regulation of

thyroglobulin and thyroperoxidase have been described in the genetic susceptibility to thyroid cancer. The literature reported FOXE1, AK023948, Premir-146a and XRCC1 as having an influence on the genetic predisposition to PTC [6]. Familial nonmedullary thyroid cancer is more locally aggressive than the nonheritable forms of thyroid cancer and involves a high rate of multicentric disease, extrathyroidal invasion, lymph node spread and an elevated recurrence rate [7, 8].

Thyroid cancer is usually a result of a transformation process based on two distinct molecular mechanisms that involve point mutation and chromosomal rearrangement. The point mutation corresponds to a single nucleotide change inside the DNA chain and the chromosomal rearrangement to a genetic abnormality with breakage and fusion of parts of the same or different chromosomes [9]. There are two classic signaling pathways that are coupled to the receptor tyrosine kinase (RTK) at the cell membrane, which transduces extracellular growth signals into intracellular signaling downstream of the two pathways represented by mitogen-activated protein kinase (MAPK) and P13KCA/AKT. The rat sarcoma gene (RAS) can couple signaling from RTK to both pathways [10•]. Genetic RTK amplifications are common, and the growth factors EGF and VEGF bound to the growth factor receptor can also stimulate these two pathways [11]. Common activating mutations in the MAPK pathway include rearranged during transfection/PTC gene (RET-PTC) mutation, RAS mutation and B-type rapidly growing fibrosarcoma kinase gene (BRAF) mutation. Common genetic alterations in the P13K pathway include RAS mutation, PTEN mutation or deletion, PIK3CA mutation or amplification, and AKT1 mutation [10•]. The molecules involved in these two pathways can be studied and detected in cytology or tumor samples and could work as potential markers for diagnosis or prognosis, and maybe to drive a target therapy for PTC.

Epigenetic transformations refer to heritable changes in gene expression that occur without any alteration in the primary DNA sequence and are implicated in some events related to tumor progression. Many genes involved in the control of cell proliferation and invasion (p16INK4A, RASSF1A, PTEN, Rap1GAP, TIMP3, DAPK, RARβ2, E-cadherin, BRAF and CITED1) as well as genes specific to thyroid differentiation (Na+/I− sym-port, TSH receptor, pendrin, SL5A8 and TTF-1) present aberrant methylation in thyroid cancer [12, 13]. DNA methylation tests may also help to identify relevant biological pathways involved in thyroid cancer development [13].

Genomic and proteonomic abnormalities can be assessed in tumor cells, tissue and blood from patients harboring thyroid cancer. The genomic markers derived from mutated or deleted genes and translocated chromosomes are studied by techniques using extracted DNA or RNA. These genomic molecular markers are best studied by cytogeneticists trying to explore some DNA molecular changes using protein chain reaction (PCR), transcriptase reverse-PCR (RT-PCR), serial analysis of gene expression (SAGE) and tissue microarray (TMA) technology. The accumulated genomic alterations are expressed in the transcriptome and then translated to the proteonome, and the related proteins can be identified, extracted and quantified by different techniques, mainly two-dimensional gel electrophoresis and the surface-enhanced laser description/ionization (SELDI)-TOF technique [14], as well as enzyme-linked immunosorbent assay (ELISA) and phage display [15]. High-throughout techniques have allowed researchers to look for and then validate several potential new biological markers in the last 10 years [16], and the efforts to define the pathogenesis and carcinogenesis of well-differentiated thyroid cancer (WDTC) yielded knowledge that was an important step in developing new protocols and algorithms for the PTC approach [10•, 17, 18].

Molecular Markers

For thyroid cancer, BRAF and RAS mutations and RET/PTC and PAX8/PPARγ rearrangements, associated with the abnormal regulation of miRNAs, constitute the most implicated molecular alterations identified in PTC [19–23]. BRAF is the most common mutation detected in PTC with a frequency of 45 % [24•] and is rare in follicular adenoma and carcinoma [16]. The mutation of nucleotide 1799 leads to a constitutive activation of BRAF kinase and chronic stimulation of the MAP kinase pathway, leading to increased cell proliferation and cell survival, which result in a carcinogenesis process [25].

The RAS oncogene (HRAS, KRAS and NRAS) encodes small GTP-ase proteins involved in signal transduction [21] and has predominance in follicular neoplasms, including follicular adenoma, follicular carcinoma and the follicular variant of PTC [22, 26].

RET/PTC rearrangements are the most common chromosomal rearrangement in thyroid cancer, and they activate both the MAPK and P13K/Akt pathways [27]. They are present in almost 20 % of PTCs in adults and are more common in radiation-induced PTCs and in carcinomas in children and young adults [28].

PAX8/PPARγ is a fusion oncogene and results from an interchromosomal translocation [2, 3] (q13, p25), leading to P13K/Akt pathway activation, and the fusion protein is involved in thyroid tumorigenesis [23]. The prevalence of PAX8/PPARγ rearrangement is higher among FTC and FTA [29].

MiRNAs are a class of small endogenous non-coding RNAs that work as negative regulators of gene expression

and are involved in some cellular processes. The miRNA test may provide a basis for further improvement in non-mutated gene cases [30], and the expression of miR-146b, miR-222, miR-34b and miR130b may be influential in PTC prognosis [31].

The Challenge of Molecular Diagnosis for WDTC

Fine-needle aspiration biopsy (FNAB) is the best method to suggest a diagnosis and provide a basis for treatment decision making for thyroid nodules [32]. It is highly sensitive in detecting malignant disease ranging from 89 to 100 %, but the specificity varies from 69 to 100 % [33]. The widely used Bethesda System for Reporting Thyroid Cytopathology is based on a six-category classification that stratifies the risk of malignancy according to the cytological findings [34]. It is well accepted that for the benign category (Bethesda category II), except for large or compressive nodules, the best approach is observation. For Bethesda categories V and VI, surgical treatment is imperative. For Bethesda category IV, surgical treatment will be of benefit mainly if the nodule is associated with some alterations on ultrasonography, but this category is controversial. The follicular lesions of undetermined significance (FLUS) group (Bethesda III) also constitutes a controversial category. [34]. An average rate of 6–55 % (mean of 24 %) of FNAB results are related to a follicular (indeterminate) pattern [35]. The risk of malignancy among the three categories included in this group, FLUS, follicular neoplasms and those suspicious for malignancy, varies from 5–10 %, 20–30 % to 50–75 %, respectively [36]. The high risk of malignancy in these latter two categories (follicular neoplasm and suspicious for malignancy) allows the clinician to carry out surgical treatment for diagnostic and therapeutic purposes. For patients showing FLUS in their FNAB, the average incidence of malignant disease ranges between 4.95 and 35 % [37–41]. Usually those patients with Bethesda category III undergo a diagnostic lobectomy, and almost 1/4 of them undergo a second surgery to complete the thyroidectomy if the first surgery confirms the cancer. Despite its feasibility, this approach may lead to unnecessary operations being performed in 75 % of patients along with the inherent risks and expenses involved in this treatment paradigm. The inclusion of a panel of biomarkers that could improve the sensitivity and specificity of the FNAB is desired in this situation and could improve the stratification of malignancy risk [22]. To date, no molecular marker or set of markers has met the requirements to answer the questions regarding the risk of cancer in the FLUS group [22]. The use of molecular markers, e.g., BRAF, RAS, RET/PTC, PAX-PPARγ or galectin-3 (GAL-3), may be considered for patients with indeterminate cytology on FNAB to help guide management [32]. Additionally, expression of some other biomarkers for PTC detection with FNAB can be studied by immunocytochemistry/immunohistochemistry (ICC/IHC) assays as follows: GAL-3, HMBE-1, cytoqueratin-19 (CK-19), TPO, VEGFR, EGFR, CITED-1 and HGF [11]. The risk of malignancy observed when one altered marker is expressed can be better evaluated when two, three or more markers are studied in a group and their data are analyzed in a mathematical model [42]. The ideal biomarker represents a test in which high sensitivity and specificity are associated with a high negative predictive value (NPV) and high positive predictive value (PPV) as well. No unique or independent biomarker has achieved this goal so far. Some gene classifiers are emerging in the literature and are being commercially tested, trying to improve the low NPV and low PPV that are common in these tests. The use of a panel of DNA/RNA mutation/rearrangement tests, studied by the microarray technique and validated by a large multicenter study, is starting to be used commercially; it is known as the Afirma gene expression classifier (AGEC) [43••]. This test has been considered to be a good tool for "ruling out" malignant tumors when associated with a negative result [17]. On the other hand, the best test to "rule in" the suspicion of a malignant thyroid tumor is the association of the expression of mutated BRAF, RAS and RET/PTC and PAX8/PPARG translocations [44••]. The real impact of those tests on clinical practice is still waiting for final validation [45].

Nikiforov et al. [44••], studying 247 patients with a FLUS pattern on FNAB tested for BRAF, RAS, RET/PTC and PAX8/PPARγ mutations, observed that the risk of histological malignancy when any mutation was detected was 88 % with an NPV of 94 %. This study was a landmark and was used as the basis to format the four-gene classifier test (miR-Inform, Asuragen Inc., Austin, TX, USA), with the intention to "rule in" highly suspicious molecular profile nodules for WDTC with Bethesda III FNAB results [17]. The number of detected mutations observed in this FNAB category is low (11.3 %) [44••], but it is in accordance with the low risk of malignancy observed in this FNAB category as well [36]. Cantara et al. [46], studying 41 cases of FNAB with indeterminate results, detected 17 % of mutations (BRAF, RET/PTC and RAS), and at final histology, all but one (follicular adenoma) were PTCs. Among 34 cases without mutations, only 1 was PTC. In nearly 50 % of cases, they did not assess RET/PTC rearrangements because of the low quality of RNA, and there was a discrepant result comparing cytology with histology in favor of tissue samples in 11.8 % (among all 234 cases studied) [46].

However, the commercially available Veracyte Afirma GEC works as a complimentary test to the four-gene classifier miR-Inform test. Alexander et al. [43••] published

a multicenter study conducted on 265 FNAB-undetermined nodules of which 32 % were malignant. This gene-expression classifier (Veracyte Afirma GEC) was able to detect 92 % of malignancies with 52 % specificity. In contrast to the 74 % rate of diagnostic surgery for the group with undetermined FNAB results, in the Afirma GEC-negative group, the frequency of surgery dropped to 7.6 % [17]. This molecular classifier is intended to "rule out" unsuspected molecular cases from surgical treatment, opening the opportunity for a watch-and-wait approach [10•] (Fig. 1).

In the literature, we can find some bias based on different ways of collecting cell samples from thyroid nodules, including half of the collected material being directed to molecular analysis, one-third or part of it to a residual washing, or material collected from a second FNAB and others [30]. Different ways to extract DNA and RNA, the storage of the material, the DNA sequencing protocol and other variables may interfere with the final result and confound comparisons among studies. The challenge of adding a DNA and mRNA extraction and miRNA test to a routine FNAB requires a specialized biomolecular laboratory able to run examinations for mutation detections, rearrangements and miRNA.

According to Ohori et al. [47], studying BRAF and RAS mutations and RET/PTC and PAX8/PPARγ rearrangements in 117 cases of FLUS category nodules examined by FNAB, the risk of cancer in the group harboring molecular alterations was 100 % compared to only 7.6 % among patients with no mutations. The addition of a molecular profile to the FNAB results improved the diagnostic accuracy in 16–42 % and allowed a more accurate selection for patients who have to undergo a surgical resection of their thyroid nodules [48, 49]. The detection of a BRAF mutation in a follicular lesion on FNAB indicates diagnostic surgery in the algorithm flow chart, which in this case is suggested to be a more extended surgery [10•, 30]. The minimal suggested surgery is a total thyroidectomy including or not elective central compartment lymphadenectomy [10•, 30] (Fig. 1). This is based on the association of the BRAF mutation with a more aggressive tumor behavior, which includes a higher frequency of lymph node metastasis, multifocality, extrathyroidal extension and others [10•, 50–52].

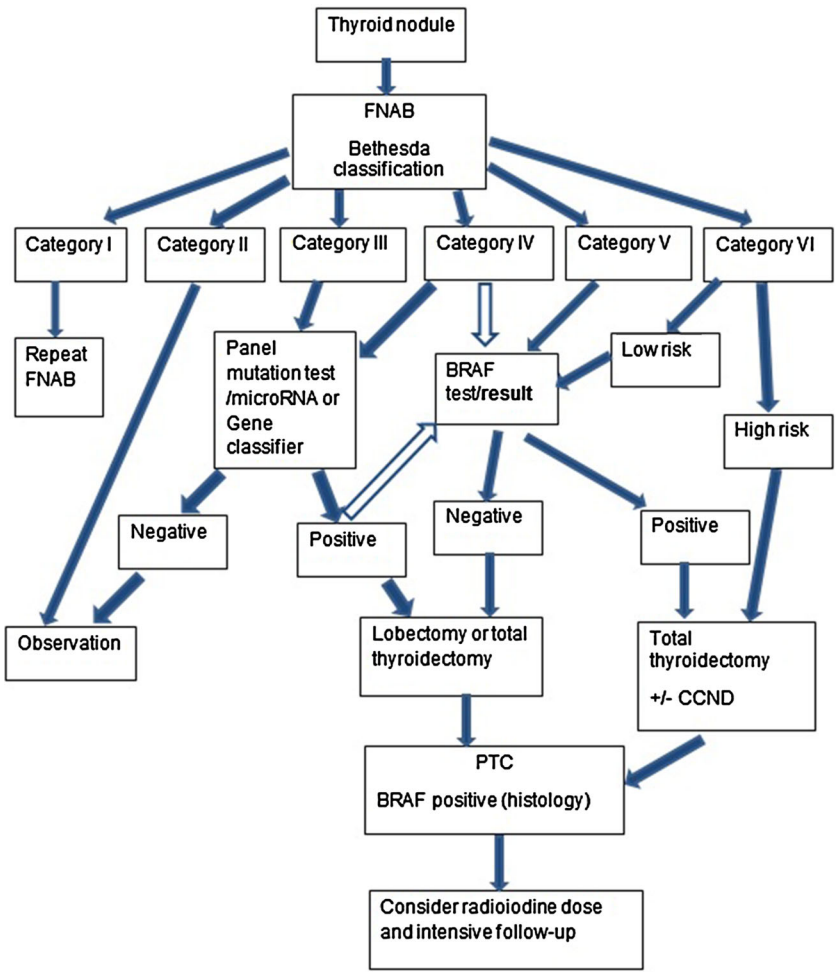

Fig. 1 Influence of the molecular marker test on the algorithm for thyroid nodule management based on the FNAB results according to the Bethesda classification. The panel mutation test includes BRAF and RAS mutations and RET/PTC and PAX8/PPARγ rearrangements associated or not with the miRNA test or the commercially available gene classifiers. The *empty arrow* points to an optional flow. For the panel mutation or gene classifier box, when positive, the previous BRAF test result (*bold*) may be used instead of performing a new test. For Bethesda category *IV*, the algorithm may permit a flow to the panel mutation test box or to the BRAF test box (*empty arrow*). *CCND* means central compartment neck dissection

In fact, about 30–40 % of differentiated thyroid cancers do not have any known molecular mutation, making the molecular test weak for diagnosis in those cases [10•]. For negative mutated cases, the inclusion of miRNA may provide a basis for further improvement [30], and the expressions of miR-146b, miR-222, miR-34b and miR130b are differentially expressed in aggressive compared with nonaggressive PTC. Otherwise, among BRAF-positive cases, miR146b overexpression is associated with a poorer outcome [31].

Immunocytochemical markers were studied in order to increase the accuracy of FNAB in the diagnosis of malignant thyroid diseases. The most studied markers were CK-19, Gal-3 and HBME-1. De Matos et al. [42] published a meta-analysis showing that the association of these three markers determined an improvement in sensitivity to 85 % and specificity to 97 % adding this immunocytochemistry assay to FNAB. They observed that Gal-3 had the better sensitivity and specificity of those three markers. Franco et al. [53] described a 10 % increase in sensitivity by combining the HBME-1 and Gal-3 immunocytochemical tests with FNAB with indeterminate results. A large-scale clinical trial failed to demonstrate the validation of immunocytochemical markers in predicting malignancy in follicular lesions [21], and the large interobserver variation has limited the widespread applicability of these tests in clinical practice [22].

To date, the best available approach including molecular analysis and FNAB, mainly for the Bethesda III/indeterminate category, appears to be using the Veracyte Afirma GEC along with the Asurgen miR-Inform thyroid test. The information provided by the miR-Inform test serves as a basis to "rule in" suspicion of malignant nodules in the surgical branch of the algorithm. An Afirma GEC-negative result will serve as a basis to exclude possible malignancy and avoid the indication for an unnecessary surgery in those in the undetermined FNAB category (Fig. 1). Obviously ongoing research will continue to look for answers to questions surrounding doubts about FNABs. The reproducibility of the molecular tests and the new protocols will validate the usefulness of this new armamentarium in the thyroid nodule diagnosis paradigm worldwide.

The Challenge of Treatment Tailored by a Molecular Profile

The use of molecular markers can help in predicting those patients most likely to have recurrent/persistent disease and to suggest when more aggressive initial treatment may play a role. Xing et al. [54] reported that finding the BRAF mutation in tests before surgery with FNAB was associated with an increased prevalence of extrathyroidal extension, thyroid capsular invasion, lymph node metastasis and recurrence/persistence of the disease and suggested that this information could be used to tailor the extent of the initial surgery. In mutated cases, a more comprehensive approach addressing the lymphatic drainage may be recommended [55].

The relationship of the BRAF mutation and other cell processes showed an association with impaired NIS expression and also a decrease in iodide-metabolizing gene expression of TSH-R, thyroglobulin and TPO [56–58]. On imaging, Barollo et al. [59] reported a decreased uptake of ^{131}I in primary and recurrent/persistent disease in BRAF mutated cases, with an inverse ability to concentrate ^{18}F-FDG in these cases. Another therapeutic suggestion based on BRAF mutation expression is to increase the radioiodine activity in patients harboring this mutation, trying to compensate for the impaired iodine uptake.

Recently, some target therapies against PTC signaling pathways have been reported. These treatments work by inhibiting signaling pathways activated by RAS and BRAF mutations and RET/PTC and PAX8/PPARγ rearrangements, and also inhibiting neoangiogenesis. Several tyrosine kinase inhibitors are being used in advanced thyroid cancer, including imatinib, gefitinib, axitinib, sorafenib, motesanib, sunitinib, XL184, pazopanib and lenvatinib [20]. The main basis of this strategy is to search for a tumor biomolecular profile before the introduction of a specific target therapy to obtain better drug response efficacy. These drugs are being studied and prescribed for advanced disease that is no longer responsive to conventional therapy, and the majority are under investigation in phase II and III clinical trials.

Some epigenetic drugs, which are targeted to the two main mechanisms of epigenetic alterations (DNA methylation and acetylation), are being studied with the aim to define the real effectiveness of these drugs for the treatment of advanced thyroid cancer. Trials including decitabine, depsipeptide HDAC 1 and 2, vorinostat, valproic acid and panobinostat have been concluded, showing that only few patients obtained complete response, nevertheless suggesting that these agents might be effective in stabilizing the disease [12].

The Challenge of Prognosis Based on a Molecular Profile

WDTC is a disease with an overall 5-year survival rate of 97.3 % and an age-adjusted death rate of 0.5 per 100,000 people [60]. Some variables are associated with a worse prognosis including advanced age, male gender, tumor size, extrathyroidal extension, race, lymph node metastasis, distant metastasis, impaired iodine uptake by the tumor/

metastasis, vascular invasion, familial history of WDTC, incomplete surgical excision and others [61–63]. Neck recurrence, usually in the form of regional lymph node metastases, occurs in up to 20 % of patients with low-risk PTC and 59 % of high-risk cases [64].

For prognostication, almost the same panel of biomarkers can be used to answer some questions regarding the recurrence rate, overall survival, specific disease survival, disease-free survival and aggressiveness for PTC cases. In the TNM clinical classification, the stage, histopathology, age, lymph node status, distant metastasis and completeness of surgical excision are the main factors that define the prognosis [63]. BRAF mutation is associated with an increased risk of lymph node metastasis, extrathyroidal extension, advanced stage AJCC III/IV and tumor recurrence [24•]; however, some studies did not find a significant association with prognosis [65–70]. BRAF mutation alters the function of the iodine symporter and is related to the decreased iodine uptake observed in BRAF-mutated cases, leading to treatment failure in recurrent tumors [56].

The incidence of central compartment lymph node micrometastatic disease in PTC varies from 20 to 90 % [71–74]. Some factors have been reported to be associated with a higher risk of lymph node metastatic spread in PTC, including multifocal tumor [71], follicular variant of PTC, extracapsular extension, thyroiditis [3], male gender, tumor size, aggressive histological type [75], MACIS score, lymphovascular invasion [76], overexpression of D1 cyclin and galectin [77], and BRAF mutation [78]. Kim et al. [79] described an increased prevalence of lymph node metastasis of around 78 % in BRAF-mutated cases and around 68 % in BRAF-non-mutated cases, with an 18 % overall incidence of recurrence. The BRAF mutation has a statistically significant association with extrathyroidal extension [54, 58, 80, 81], and extrathyroidal extension carries an increased risk for recurrence of PTC and papillary thyroid microcarcinoma (PTMC) [82] and for decreased survival [61]. Another important influence of the BRAF mutation on the PTC prognosis is that this mutation is associated with a lowered expression of NIS and TSHR. These findings provide further evidence that BRAF might be associated with a more aggressive phenotype and less differentiated state because of decreased expression of iodide-metabolizing genes [57].

In paraffin-embedded tissue samples from patients with thyroid tumors of follicular cell origin, some immunohistochemical methods using monoclonal antibodies, e.g., against CK19, predicted a more aggressive cancer [83]. Complementary immunohistochemical studies including nuclear and cytoplasmic Ep-ICD expression and loss of membranous EpEx were found to correlate positively with metastasis in PTMC patients [84]. He et al. [85] reported a nuclear Ep-ICD accumulation and decreased membrane EpEx expression in aggressive PTC with 70.2 % sensitivity and 83.9 % specificity for nuclear Ep-ICD for differentiating aggressive from non-aggressive PTC.

RET/PTC studies did not find a strong association between the rearrangements and PTC prognostic factors. Adeniran et al. [86] reported RET/PTC rearrangements presented at younger age and had predominantly typical papillary histology, frequent psammoma bodies and a high rate of lymph node metastases, but these findings might be related to the higher frequency of lymph node metastases observed in young patients, and it is not an important prognostic factor for this age group [32]. RET/PTC rearrangements are quite frequent in radiation-induced PTC as well [87].

RAS mutation can be associated with a more aggressive outcome in PTC with an increased risk for distant metastasis and mortality [88], but these mutations can be found in benign and malignant thyroid tumors, limiting their significance for diagnosis and prognosis [89]. It seems that RAS mutations are related to a molecular profile that indicates a tendency to encapsulation of the tumor with a less infiltrative behavior [90]. However, Garcia-Rostan et al. [91] reported the association of RAS mutations with aggressive thyroid cancer phenotypes and poor prognosis comparing RAS-positive mutated cases with negative ones.

PAX8/PPARγ rearrangements are follicular thyroid cancer-specific gene mutations expressed in follicular carcinoma and adenoma and do not appear in classical PTC, but could be found in the follicular variant of PTC [50]. In follicular thyroid cancer, PPARγ staining also shows an association with favorable prognosis and may have a role in risk stratification [92].

Conclusion

The molecular alterations in signaling pathways involved in thyroid carcinogenesis appear to be playing an increasing role in thyroid nodule diagnosis. The addition of molecular tests to information from FNAB results improved the accuracy for undetermined cases. Some mutations are related to a more aggressive behavior, mainly the BRAF mutation, and for BRAF-positive cases, it seems reasonable to escalate the surgical treatment and to consider a higher adjuvant radioiodine dose. In advanced cases, a molecular tumor profile may suggest the use of some target therapies based on the expression of specific gene alterations. The molecular prognostic markers are playing an important role in determining the PTC prognosis based on the fact that some indolent tumors, mainly PTMC, may have an aggressive biological behavior and could be tailored for a more comprehensive approach and intensive follow-up.

Compliance with Ethics Guidelines

Conflict of Interest Gilberto Vaz Teixeira and Claudio Roberto Cernea declare no conflicts of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by the authors.

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- •• Of major importance

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