Currently Used and New Molecular Markers for Thyroid Cancer Diagnosis

Hatem A Hejaz♦, PhD, Isra' Abuzaina, PhD, Reem Naser Aldeen, PhD, Sondos Saad, PhD

College of Pharmacy and Medical Sciences, Hebron University, Hebron, Palestine

Abstract

Thyroid cancer is highly common all around the world. Its prevalence has rapidly increased over the last 30 years in the United States and other developing countries. Fine-needle aspiration biopsy has become the cornerstone of thyroid nodule diagnosis, whose general reliability is outstanding; however, some aspirates have shown undetermined cytological findings that do not provide a definitive malignancy diagnosis. At least 70 molecular and genetic markers in thyroid nodules have been analyzed in an effort to identify molecular markers to differentiate malignant and benign (BN) thyroid nodules. The present review focused on the currently used markers in thyroid cancer diagnosis. A rising number of studies have investigated immunohistochemical markers, such as galectin-3 (GAL3), cytokeratin 19 (CK19), hector battifora mesothelial-1 (HBME-1), and thyroid peroxidase, along with DNA alterations, including mainly BRAF (B-Raf proto-oncogene, serine/threonine kinase) and RAS (Ras proto-oncogene, GTPase) point mutations, Telomerase reverse transcriptase mutations, ret proto-oncogene/papillary thyroid carcinoma and PAX8/PPARG rearrangements, and miRNA signatures and circulating tumor cells for thyroid cancer diagnosis. Although certain markers are promising for differential diagnosis, due to limitations of the substantial prevalence of BN thyroid tumors, none of them is specifically definitive to a large extent. Herein, we also discussed the studies that have supported the use of combinations of several markers, like KIT, TC1, miR-222, and miR-146b combination, as well as GAL3, CK19, and HBME-1 combination, in enhancing the diagnostic accuracy in differentiating malignant and BN tumors.

Keywords: Thyroid cancer, Thyroid nodule, Molecular marker, Prognostic marker

♦Corresponding Author:
Hatem A Hejaz, PhD
College of Pharmacy and Medical Sciences, Hebron University, Hebron, Palestine
Tel:+970-2-2220995, Ext: 290
Fax:+970-2-2229303
E-mail: hhejaz@hebron.edu

Introduction

Thyroid cancer (TC) arises from thyroid gland follicular tissue. Within the thyroid parenchyma, there are two kinds of tissues: the follicular tissues and the supporting tissues (also named the C tissues). Even though not generally violent, about 10 to 15% of these cancers will ultimately transform into more violent versions. Cancers usually originating from follicular cells are called differentiated thyroid carcinomas (DTCs). Roughly 85%

of cases are found with DTC, and after management, they have an impressive prognosis.[1]

TC has no identified etiological reasons, although exposure to radiation has been concerned for many decades. Even though the occurrence of TC after exposure to radiation is higher, the physiological activity of cancer is identical in both radiation-exposed and non-radiation-induced thyroid carcinomas. Thus, while exposure to radiation is necessary to cause cancer, it does not tend to play a role in deciding the malignancy's aggressiveness.[1,2]

Over the last 30 years, the prevalence of TC has steadily increased in the (U.S.) and other developing countries.[1,3,4] A Surveillance, Epidemiology, and End Results System (SEER) analysis found that there was a triple rise in the prevalence rates between 1975 and 2009, from 4.9-14.3 per 100,000 people, while death rates remained relatively stable at ~0.5 deaths per 100,000 people.[1] This rise was mainly in minor (< 2 cm) papillary carcinomas, with a total of four times higher incidence of TC in females than that in males. Based on previous studies assessing the SEER system, TC has risen annually by 3% in the U.S. since the 1990s.[1,5] The prevalence of TC in people with high socioeconomic backgrounds and those in urban areas has increased.[6]

Among the surgically resected indeterminate thyroid nodules, 10%–40% are believed to be malignant. Several surgical procedures for benign (BN) thyroid nodules are performed as a result. On the other side, a second operation is typically performed to remove the residual thyroid lobe for those patients who have undergone surgical lobectomy and were found to have a tumor greater than 1 cm. Additional diagnostic markers are therefore required to direct the management of patients with indeterminate thyroid nodules to reduce the incidence of undue diagnostic lobectomies and two-step surgeries.[7,8]

The rationale for using molecular markers in determining indeterminate thyroid nodules is to prevent unnecessary surgery for the patient while retaining a high degree of sensitivity for cancer detection. Thyroid surgery is associated with a 2%-10% risk of surgical complications and a 10%-20% chance of lifelong supplementation of levothyroxine following lobectomy.[9]

A biomarker is characterized as a factor that can be accurately measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological reactions to a given treatment. Biomarkers could be used to classify people at risk of cancer, diagnose (early) disease, track patient response, assess prognosis, diagnose recurrence, and predict reactions to particular therapeutic approaches.[10]

Initially, the term "tumor marker" was used to denote a protein in the blood capable of indicating tumor involvement or suspicion. Currently, the usefulness of tumor marker has been extended to meet a specific criterion which includes the following: (a) detection of subjects at the likelihood of developing a particular type of tumor; (b) early detection of metastasis of a primary tumor; (c) contributing to tumor histotype; (d) assisting with the prognosis; (e) tracking cancer progression, either primary or metastatic; f) the therapy response monitoring; (g) better explaining the pathogenesis of tumors; (h) proposing diagnostic or therapeutic measures linked to the tumor marker itself or molecular events associated with tumor markers.[11]

Results and Discussion
Diagnosis of TC
There are different tests and procedures to diagnose TC, including physical examination, blood tests, ultrasound (US) imaging, removing a sample of thyroid tissue, other imaging tests (computed tomography (CT), magnetic resonance imaging (MRI), and nuclear imaging tests), and genetic testing. The actual diagnosis of TC is made with a biopsy, in which cells from the suspicious area are removed and looked at in the lab. However, all the new molecular markers in TC have been discussed.

Fine needle aspiration biopsy (FNAB)
The first-line diagnostic techniques for thyroid nodules are high-resolution ultrasonography and FNAB. The thyroid ultrasonography is convenient and non-invasive, but the US characteristics are not sufficiently sensitive to detect all TCs.

Therefore, thyroid aspiration biopsies are the most effective diagnostic tool for TCs.[12-14]

To date, fine needle aspiration (FNA) biopsy has been the most successful approach to thyroid nodules analysis and has been considered as the gold standard diagnostic tool for TC. FNA has a high level of sensitivity and specificity in distinguishing BN from malignant thyroid lesions. However, the key drawback of this diagnostic procedure is the indeterminate outcome of about 20%-30% of samples, causing difficulties for these patients to be handled efficiently. However, in the final histology, only 10%-30% of indeterminate thyroid nodules are found to be malignant.[8,15-17]

The determination of whether a thyroid nodule is BN or cancerous is an important diagnostic step. A local anesthetic can be injected into the skin before biopsy, to numb the area. The doctor sticks a small needle into the nodule and extracts some fluid and cells. They repeat the process twice or three times in order to get samples from different nodule areas, as illustrated in figure 1. A report is then provided about the results of this test by the cytopathologist.[18,19]

The FNA diagnostic categories are as follows: (I) non-diagnostic or unsatisfactory (smears were deemed to be non-diagnostic, when a thyroid FNA sample failed to meet the required adequacy criterion for at least six groups of well-visualized follicular cells with at least 10 cells per group, preferably on a single slide, colloid, or blood only, and the estimated malignancy risk was 1%–4%); (II) BN (the lesions were categorized into this group, if diagnosed or identified as a colloid nodule, multinodular goiter, and lymphocytic or granulomatous thyroiditis, and also if the aspirate showed only BN follicular cells, the risk of malignancy was 0%–3%); (III) atypia of undetermined significance or follicular lesion of undetermined significance (AUS/FLUS) (within this group, the lesions were identified as diagnosed or satisfactory with 'atypical cells/atypical follicular cells' followed by a note indicating that neoplasm could not be removed and in this case it is suggested to repeat FNA of the lesion, cancer risk was 5%–15%); (IV) follicular neoplasm or suspicious for follicular neoplasm (FN/SFN) (if diagnosed or identified as having high follicular cellularity with predominant microfollicular shapes, scant colloid, the lesions were in this group. These often included lesions showing primarily hurthle cells and those treated as a suspect for hurthle cell neoplasm, the risk of malignancy was 15%–30%); (V) suspicious for malignancy (within this group, the lesions were marked, if they were diagnosed or identified as the suspect of papillary, medullary, or metastatic carcinoma or lymphoma. Smears were predominantly cellular in this category with crowded cell groups showing nuclear and cytoplasmic pleomorphism with some rare single atypical cells, the risk of TC was 60%–75%); (VI) malignant: (the lesions belonging to this group were diagnosed with type classification as essentially malignant, the risk of TC was 97%–99%).[15,19,20]

In this review, we provided the currently used markers in TC diagnosis. A rising number of studies have investigated immunohistochemical markers (such as galectin-3 (GAL3), cytokeratin 19 (CK19), hector battifora mesothelial-1 (HBME-1), and thyroperoxidase (TPO), DNA alterations (including mainly B-Raf proto-oncogene, serine/threonine kinase (BRAF), and Ras proto-oncogene), GTPase point mutations (RAS), telomerase reverse transcriptase (TERT) mutations, ret proto-oncogene/papillary thyroid carcinoma (RET/PTC), and PAX8/PPARG rearrangements,[21,22] along with miRNA signatures and circulating tumor cells for TC diagnosis.[20] Although certain markers are promising for differential diagnosis, due to limitations of the substantial prevalence of BN thyroid tumors, none of them is specifically definitive to a large extent.[23]

Molecular genetics of TC

The TC-associated genetic changes have been well described. Various studies have been conducted in multiple laboratories and established the driver mutations for most thyroid tumors. The current large-scale sequencing schemes have detected genetic changes in many of the lasting thyroid tumors and offered an overview of the

evolving landscape. Such results played a significant role in shaping and developing the thyroid tumor classification for representing the histological, molecular, and behavioral characters.[24] For note, not all of a cancer genome's somatic mutations are concerned with cancer initiation because others are the consequences of carcinogenesis. A mutation is a consequence of oncogenesis in cancer stem cells and is positively selected in the tissue microenvironment where cancer starts and is not required to sustain final cancer.[25] The combined study of genomic variations, gene expression, and DNA methylation of TC has shown that different driver mutation classes contribute to different pathologies with distinct characteristics of signaling and differentiation. Most of the changes seen comprised genes that work in the pathways of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K), as shown in figure 2.[21,26] Most TCs harbor mutations along the MAPK cellular signaling pathway. This pathway carries signals of growth from the cell membrane to the nucleus and takes a core part in regulating cell proliferation.[27] In particular, molecular analysis has concentrated on a group of somatic gene changes in the MAPK pathway, commonly present in thyroid carcinomas. These changes include

point mutations of the genes BRAF and RAS, and chromosomal rearrangements of RET/PTC and PAX8/PPARÿ V600E mutation in the BRAF gene was found to be the most common genetic occurrence in PTC in 40 to 45% of cases.[28]

Alterations involving the PI3K/AKT signaling pathway are likely to play a role, particularly in the later stages of tumor progression. These changes, including those involving the TP53 and CTNNB1 gene, occur with variable prevalence in poorly-differentiated and anaplastic carcinomas. RET point mutations are common in medullary thyroid carcinomas. In addition, PI3K-activated AKT may phosphorylate a series of downstream target proteins, including Bad, caspase 9, forkhead, Par-4, p2l, and mammalian rapamycin (mTOR) target to activate or inhibit their actions, which ultimately fosters cell survival. Thus, AKT is considered as an antiapoptotic regulator.[28,29] Such pathways may collaborate, encourage, antagonize, or interact to form a complex regulatory network. Dysfunction of this network may increase TC growth, progression, invasion, and metastasis.[29,30] Many of these mutations are associated with distinct phenotypical features of tumors, and some of them serve as markers of more aggressive tumor behavior. The current molecular techniques allow the detection of these genetic alterations
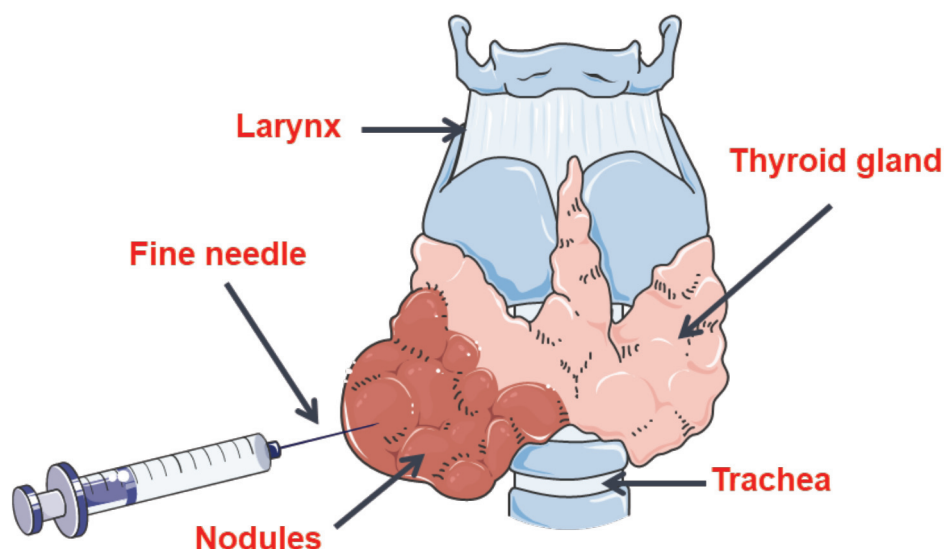

Figure 1. This picture illustrates a fine needle aspiration for thyroid nodules.[19]

in thyroid FNA samples and surgically removed samples, offering useful information for the diagnosis and management of patients with TC. Moreover, many of these mutations, especially those activated the MAPK pathway, are diagnosed for targeted therapy of TC.[27,29,31]

The left side of figure 2 displays the MARK pathway, which is stimulated by a mutation in most thyroid carcinomas (cancer initiation). Such events are thought to trigger the development of TC and contribute to altered gene expression, encouraging cell proliferation, cell replication, angiogenesis, and loss of differentiation. The right side demonstrates the altered pathways in advanced cancers of the thyroid, which are known to facilitate the development of tumors (cancer progression). This includes the PI3K–mTOR pathway, p53 tumor suppressor, and TERT promoter modifications. Blue boxes reflect the factors that have been approved by the U.S. Food and Drug Administration, for which specific therapies are available. PTEN acts as a tumor suppressor gene through the action of its phosphatase protein product. This phosphatase is involved in the regulation of the cell cycle, preventing cells from growing and dividing too
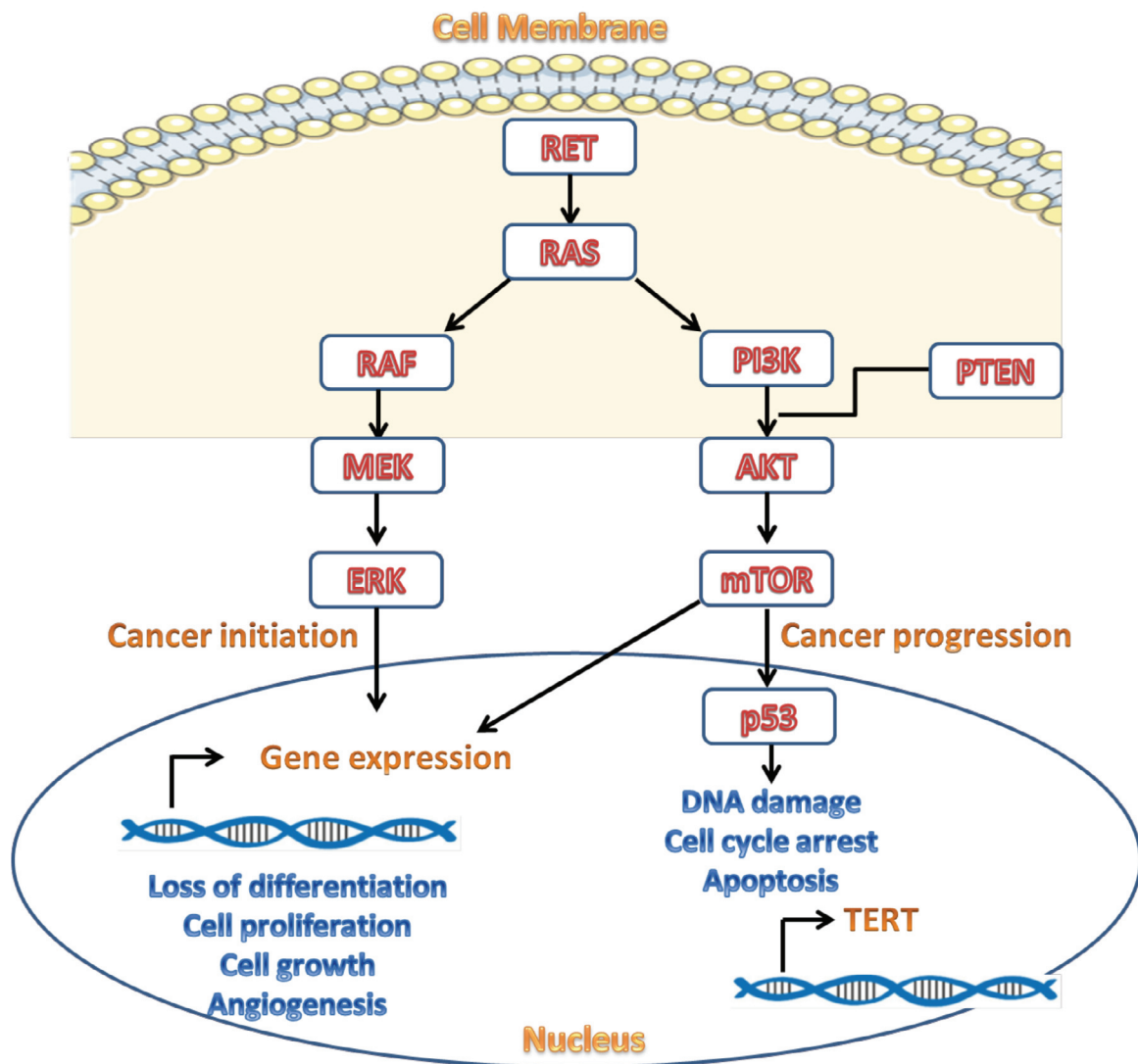

Figure 2. Thyroid cancer pathways; the diagram shows the key molecular signaling pathways involved in thyroid cancer.[27]
RET: Ret proto-oncogene; RAS: Ras proto-oncogene; RAF: Raf proto-oncogene; MER: Mitogen-activated extracellular signal-regulated kinase; ERK: Extracellular signal-regulated protein kinase; AKT: V-akt murine thymoma viral oncogene homolog; mTOR: Mammalian target of rapamycin; PI3K: Phosphatidylinositol 3-kinase; TERT: Telomerase reverse transcriptase; PTEN: Phosphatase and tensin homolog; p53: Phosphoprotein p53 (Tumor protein p53)

rapidly; it is a target of several anticancer drugs.[27]

Classification of TCs based on their cell origin and the inheritance pattern

Generally, TCs can be classified in two ways, either based on their cell origin or the inheritance pattern. TCs could be categorized according to their origin cell into two classes: Endoderm-derived TC and Neural-crest C-cell derived TC. Endoderm-derived follicular cell cancers consist of DTC [which in turn includes papillary TC (PTC) and follicular TC (FTC)], anaplastic TC (ATC), and poorly-differentiated TC (PDTC). PTCs are genetics disease-causing variants of BRAF V600E (60% of disease-causing variants), RAS (15% of disease-causing variants), RET, EIF1AX, PPM1D, CHEK2, NTRK fusion, ALK fusion, and DICER1. FTCs, including Hurtle cell cancer, is genetics disease-causing variants of RAS, PAX8-PPARγ fusion gene. PDTCs are genetics disease-causing variants of BRAF, RAS,
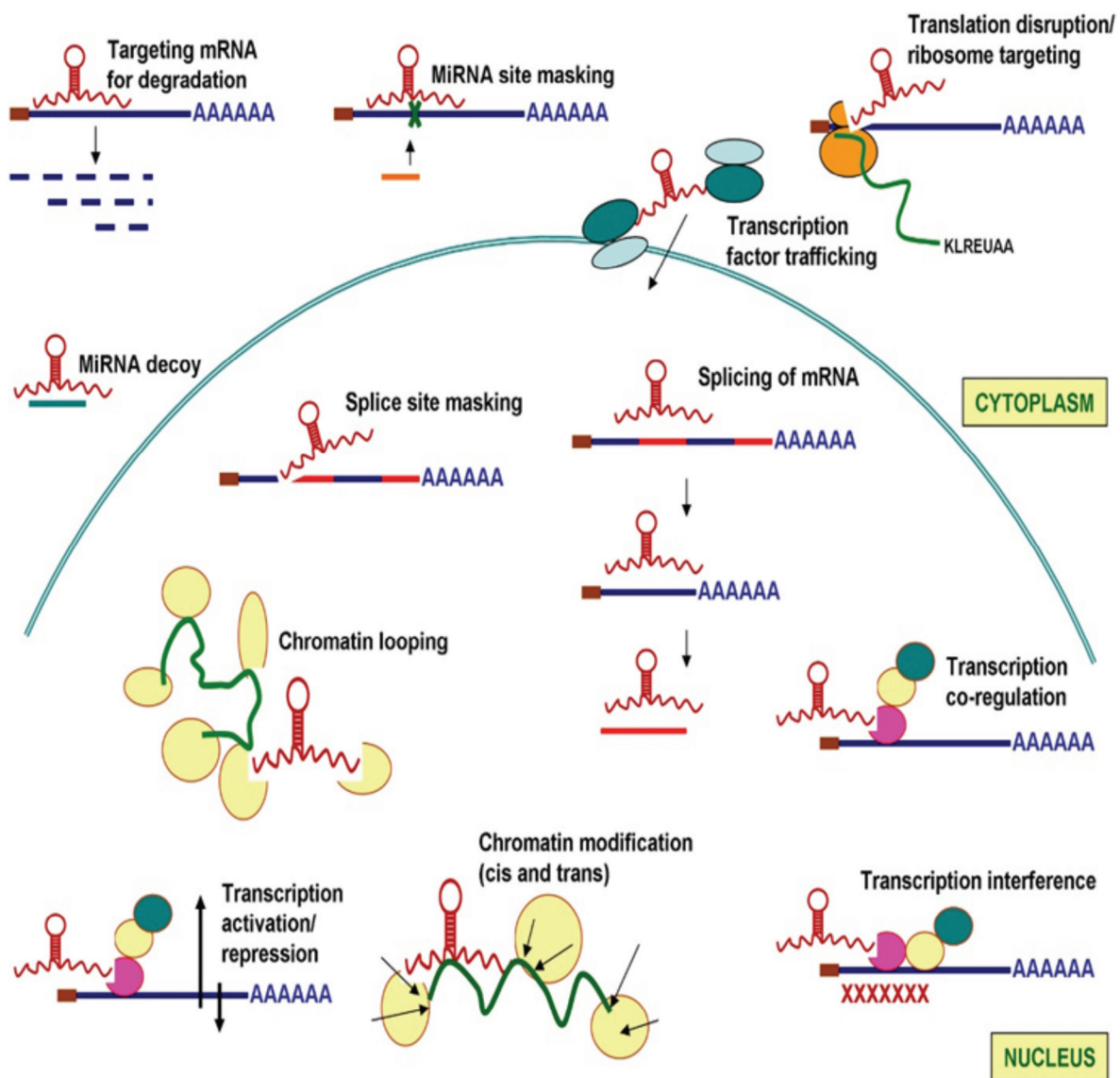
Figure 3. lncRNAs and their functions; this illustration shows various functions of lncRNAs. The diagram in figure 3 shows various lncRNA roles. In the nucleus, lncRNAs are involved in looping chromatin, modifying chromatin in both cis and trans, regulating transcription, and splicing mRNA. lncRNAs target mRNA for cytoplasm degradation or defense, transcription factor trafficking, miRNA site concealment, and translation disruption/ribosome targeting.[90]

TERT, and EIF1AX. Meanwhile, ATCs are genetics disease-causing variants of B-Raf proto-oncogene, serine/threonine kinase, RAS, TERT, EIF1AX, TP53, CTNNB1, PIKC3A, PTEN, and AKT1. Other disease-causing molecular changes are found in the SWI/SNF complex and histone methyltransferases. Medullary TC (MTC) is derived from C-cells originated in the neural crest. Based on the inheritance pattern, TC may occur either sporadically (non-inheritable) or as part of a familial (inheritable) or genetic disorder.

Familial syndromes are divided into two classes of familial medullary thyroid carcinoma (FMTC), derived from calcitonin-producing C cells, and familial non-medullary thyroid carcinoma, derived from follicular cells. The familial form of MTC is usually a component of multiple endocrine neoplasias (MEN) IIA or IIB or presents as pure FMTC syndrome. The histopathological characteristics of the tumors in patients with MEN syndromes are identical to those of sporadic tumors, except for bilaterality and multiplicity of tumors. The genetic procedure in the familial C-cell-derived tumors is common, and genotype-phenotype correlations are well-determined. However, the issue concerning a familial propensity of non-medullary thyroid carcinoma is only now beginning to arise. Even though the majority of papillary and follicular thyroid carcinomas (FTCs) are occasional, the familial forms are infrequent and could be classified into two groups. The first group is the familial syndromes which are characterized by the prevalence of non-thyroidal tumors, such as familial adenomatous polyposis and PTEN-hamartoma tumor syndrome, among others. The second one is the familial syndromes characterized by the prevalence of PTCs, such as pure familial PTC (fPTC), fPTC linked with papillary renal cell carcinoma, and fPTC with multinodular goiter.[32] PDTC is a separate TC histotype. Regardless of infrequency, it is the main cause of death from non-anaplastic follicular cell-derived TC. Most TC types are highly curable. Actually, the most frequent types of TC (PTC and FTC) are the types that are more likely to be cured. Both papillary and follicular cancers have a high cure rate (more than 98%) in young patients (below 50 years of age), if treated properly.[33]

DTC

Approximately 85% of all TCs are PTCs, while FTC and Hurtle cell cancer together accounts for up to 5% of all TCs. The genetic landscape of PTC is heterogeneous, made up of mutually unique mutations involving the pathway of MAPK. Certain genetic mutations (RAS, RET / PTC, and BRAF) are the characteristics of PTC. Despite low accuracy (about 50%), BRAF mutations that were not discovered in normal thyroid tissue represent the most specific PTC mutations. These mutations are associated with avidity loss of radioiodine, increased nodal metastases, poor survival, reduced thyroid-specific gene expression (such as the thyroid-stimulating hormone receptor and TPO), and distinctive of differentiated PTC; since BRAF mutations are considered to be mutually exclusive in PTC.[32-36]

RAS genes (HRAS, KRAS, and NRAS) are G proteins that signal the pathways of both MAPK and PI3K/AKT. Typically, point mutations in the RAS genes occur in codons 12, 13, and 61, and are noticed in 40%–50% of follicular cancer and 10%–20% of papillary TC. RAS-like PTCs include RAS as the primary disease-causing variants (~15% of all PTCs) and are characterized as follicular morphology PTCs and low MAPK pathway signals. Other different disease-causing variants have been also identified, such as the NTRK fusion genes, RET, EIF1AX, PPM1D, and CHEK2.[34]

FTC is characterized by translocations and fusions of several genes (PAX8 and PPAR gamma) and by unique protein expression. Gains of genomic copies and PIC 3CA amplification, as well as RAS mutations, are commonly found in FTC. The mitogen-activated protein (MAP) kinase and P13K / AKT pathways in TC can be triggered by almost all these genetic mutations. These pathways are also triggered by aberrant methylation of essential genes belonging to the tumor suppressor and the thyroid. These genetic markers become therefore useful in the development of therapeutic strategies targeted at the MAPK and P13K / AKT pathways.[11,37]

MTC

MTC accounts for nearly 3%–5% of all thyroid malignancies, which is derived from parafollicular calcitonin-producing C-cells of the thyroid gland. It rises primarily as a sporadic tumor in about 60% of cases, and is inherited in 40% of cases. MTC is the main disease feature of type 2 multiple endocrine neoplasia (MEN 2), MTC is an autosomal mostly inherited cancer condition caused by RET mutations in the germline alterations in the RET protooncogene.[39]

Medullary thyroid carcinoma has been reported in conjunction with elevated serum calcitonin levels through physical examination. Diagnosis of MTC includes cytological and histological confirmations. US, CT, and MRI are used to determine the size of the tumor. MTC classification is based on a method of pathologic tumor, node, and metastasis.[10,40]

In the pathogenesis of sporadic MTC, pathogenic variants of RET (40%–50%) and RAS (20% and STK11 (10%–20%) genes are involved. Remarkably, the disease-causing forms of RET and RAS (HRAS and KRAS) are usually mutually exclusive.[41] The somatic M918T disease-causing variant in RET oncogene is the most common type of alteration seen with sporadic MTCs (> 75% of the variants of RET disease-causing variants), as well as a major germline disease-causing variant seen with multiple endocrine neoplasia type2 (MEN2) B syndromes.[34,42,43]

Anaplastic thyroid carcinoma (ATC)

Out of all thyroid malignancies, ATC is the most severe one and has the worst prognosis, with a median survival rate of 5–12 months and a survival rate of 20%–40% a year.[44] ATC cancers are generally categorized as stage IV disease irrespective of the tumor burden and metastasis presence or absence. Based on the level of invasion of the underlying tissue layers, these are subclassified as IVa, IVb, IVc, and IVd.[43] The last detailed set of guidelines for ATC management was developed and released by the American Thyroid Association (ATA) in 2012, and the ATA is at the moment, developing a new set of comprehensive guidelines.[45] ATCs harbor multi-gene mutations, including BRAF, RAS, TP53, EIF1AX, CTNNB1, and genes involved in the AKT-mTOR pathway, the SWI / SNF complex, and histone methyltransferases. Nevertheless, the major driving genes are commonly B-Raf proto-oncogene, serine/threonine kinase and Ras proto-oncogene, and GTPase.[34,45]

PDTC

PDTC is an uncommon primary thyroid tumor, affecting 4 to 7% of all cancers of the thyroid. It plays an intermediate role, morphologically and biologically, between well-differentiated thyroid carcinoma (DTC, papillary and follicular carcinoma), and undifferentiated thyroid carcinoma (ATC).[45] PDTC includes BRAF, RAS, TERT, and EIF1AX genetic mutations.[34] Recent studies have shed light on a new molecular marker associated with the genetic alteration. This molecular marker is TERT.

TERT

Increased proliferation is a key feature of malignant tumors and thyroid tumor is no exception to this principle. To prevent the sequential shortening of vital, chromosomal content that follows undue excess duplication, eukaryotic genomes are coated with telomeres-repetitive, non-coding series at the chromosomal endings. These telomeres are preserved by telomerase, an enzyme mainly produced by both RNA and protein ingredients; the latter is known to be encoded by TERT. In human cancer, elevated TERT expression and telomerase activation have been shown as recurring events, leading to stimulation of telomerase, bypassed senescence, and consequential immortalization. Although the leading cause of upregulation of TERT remains unclear, two persistent TERT promoter point mutations (C228 T and C250 T) have been identified in intermittent and hereditary forms of TC over the recent years. Since then, these mutations have been reported in different types of tumors, including TC subsets. The mutations are believed to increase TERT expression in the promoter area and are therefore thought to give a critical advantage in tumors with a significant reproduction.[47,48]

In TC, TERT precursor mutations are closely associated with more developed disease types,

such as PDTC and ATC, and also in well-differentiated cases of PTC and FTC, with undesirable diagnostic variables, like older surgical age, extrathyroidal expansion, lymph node, and distant metastasis. The C228 T mutation is the most prevalent mutation in TC, which occurs in approximately 9 out of 10 patients with a TERT gene mutation. The explanation for this selectivity is not clear, but may stem in principle from different expressional habits of transcription-factor exhibiting a preference for the location of C228 in TC. Other TERT gene mutations alongside C228T and C250T are very rare.[47,48]

In addition to the above-mentioned promoter mutations, other changes in the TERT gene have been identified as frequent incidents in human cancer. In particular, copy number benefit of the TERT gene located at chromosome 5p15 and abnormal methylation of two separate parties inside the TERT promoter have been shown to be correlated with changed TERT gene expression. These incidents were reported for different cancers, like malignant TCs. High incidence of methylation, compared with normal cells, in TERT promoter regions (578 - 541) base pairs (bp) (entitled "region A") was identified as a common occurrence in MTC; this pattern has been associated with poor patient survival.[47]

A study conducted by Liu and Xing[49] on 308 FNAB samples pre-operatively collected from thyroid tumors with post-operatively verified pathological diagnosis. On top of the BRAF V600E mutation, they studied the two TERT promoter mutations using direct DNA sequencing. TERT gene mutations were reported in 0.0% (0/179) of BN thyroid tumors and 7.0% (9/129) of differentiated thyroid tumor thyroid nodules, indicating a 100% specificity in diagnosis and 7.0% sensitivity. Furthermore, it indicated an increased sensitivity to 38.0% (49/129) when used in combination with BRAF V600E. Many TERT promoters on FNAB were cytologically undetermined for positive mutation nodules. They reported that about 80% of the TERT gene mutation-positive thyroid tumors were TCs with violent pathological behaviors, such as extrathyroid invasion, metastasis of the lymph node, marginal metastasis, relapse of condition, or loss of patients. Therefore, a positive TERT gene mutation test not only identifies a thyroid nodule to be cancerous, but also pre-operatively detects severe potential cancer. They concluded that checking TERT gene mutations on FNAB improves and reinforces the existing molecular-based methods of thyroid nodule and TC diagnosis.[49]

Another study conducted by Liu and Xing[50] identified that TERT gene mutations are correlated with violent features of the thyroid tumor, tumor relapse, and patient death, as well as BRAF V600E. The coexistence of BRAF V600E and TERT gene mutations had a significant synergistic effect on PTC's aggressiveness, which included a significantly increased relapse of tumors and patient death; whereas, the presence of individual mutation has a minimal effect. Hence, TERT with gene mutations reflects a popular new oncogene in thyroid carcinoma, and the mutations offer new TC diagnostic and prognostic genetic markers that, when combined with BRAF V600E mutation, appear to be medically beneficial in TC management. A similar synergistic impact could also be observed in TC when TERT promoter mutations are used in combination with RAS mutations, probably via activation of the PI3 K pathway. Such clinicopathological findings clearly support the significant role of TERT gene mutations in angiogenesis and TC development, which is well confirmed by earlier reports on the related forms of TERT differential expression in BN and malignant thyroid nodules.[50]

Molecular diagnostic tests

The mutational analysis of single genes in indeterminate or suspect thyroid nodules has not proved conducive to making management-related decisions. Moreover, up to 35% of thyroid nodular fine needle biopsy (FNB) procedures yield an indeterminate result that includes Bethesda category III (atypia of undetermined significance/follicular lesion of undetermined significance [AUS/FLUS]) or category IV (FN/SFN). Thus, the use of panels of molecular markers has been focused on. The 2015 American Thyroid Association Management Guidelines for

Adult Patients with Thyroid Nodules and DTC suggest the need for molecular testing in thyroid nodules with indeterminate cytological tests to facilitate malignancy risk assessment.[9,51-53]

Afirma gene expression classifier (GEC) and Thyroseq V2 are the most commonly used molecular tests in clinical applications.[53] The Afirma GEC was introduced in January 2011 and measured RNA expression to identify BN thyroid nodules. The GEC demonstrated high sensitivity and negative predictive value (NPV) of 94%-95% between the thyroid nodules Bethesda III and IV. The GEC, however, had a generally low specificity of about 50% and a positive predictive value (PPV) of about 38%. Furthermore, it functioned poorly with Hürthle cell (or oncocytic) rich aspirates as the majority of GEC suspicious nodules have been shown to be BN after surgery.[54]

The updated Afirma Genomic Sequencing Classifier (GSC), existing commercially since 2017, uses RNA sequencing and machine learning algorithms of the next generation to leverage more enriched, previously undetectable genomic details.[54]

The GSC demonstrated improved test specificity, while retaining its high sensitivity and NPV, by using the same prospective, blinded, multicenter cohort used to validate the GEC. The GSC also demonstrated improved oncocytic (Hürthle cell) lesion sensitivity through the introduction of a similar classifier.[9]

ThyroSeq v2 is a DNA-sequencing panel of the next generation, which detects genetic changes associated with TC. It was designed to detect mutations in > 1000 hotspots of 14 genes linked to TC (AKT1, BRAF, CTNNB1, GNAS, HRAS, KRAS, NRAS, PIK3CA, PTEN, RET, TP53, TSHR, TERT, and EIF1AX) and 42 gene fusion or rearrangement forms are known to occur in this type of cancer (RET, PPARG, NTRK1, NTRK3, BRAF, and ALK).[51,55]

Various recent single-institutional studies have been published evaluating the output of ThyroSeq v2.[55-57] However, these studies are constrained by their heterogeneous exclusion criteria and methods of analysis as well as their single-institutional nature.

ThyroSeq v3 is a next-generation sequencing assay based on DNA and RNA, which analyzes 112 genes for some genetic mutations, including point mutations, insertions/deletions, gene fusions, alterations in copy numbers, and abnormal gene expression. Additionally, the genomic classification is used to distinguish malignant lesions from BN lesions. This was confirmed with known surgical follow-up in 238 tissue samples and 175 FNA samples. Certain studies have been conducted in this regard with analytical performance.[58]

In clinical validation tests, ThyroSeq v3 has a sensitivity of 93.9% and an NPV of 97% for AUS/FLUS and FN / SFN (with FNHCT / SFNHCT) diagnoses. Owing to these robust values, ThyroSeq v3 is now considered to be a rule-out study, and the analysis of its BCR and other molecular test results distribution is believed to improve the understanding of its efficiency.[59]

MicroRNAs panels

Several molecular changes found in TC can be used as biological markers for diagnosis, prognosis, and treatment. For a better diagnosis of TC, new molecular signatures are required. MicroRNAs are one of these new markers. miRNAs are small non-coding endogenous RNAs, comprising approximately 19–25 nucleotides in length, which are involved in controlling gene expression. Moreover, miRNAs are highly essential in biological and metabolic pathways, such as developmental process control, signal transduction, cell maintenance, and differentiation. The dysregulation can therefore expose individuals to malignancies. miRNA expression is dysregulated in various types of tumors, especially TCs, and can be the cause of tumor initiation and progression.[14,20,59-61]

miRNAs have also been found in the blood circulation, with their detections in the serum/plasma, erythrocytes, platelets, and nucleated blood cells. The serum/plasma miRNAs are very stable; as a result, the possibility of the use of the extracellular circulating miRNAs as biomarkers for TC was explored to differentiate the patient from normal healthy individuals.[62] There is ample evidence that miRNAs play a

significant role in thyroid carcinogenesis. Therefore, miRNAs have been considered as effective diagnostic and prognostic markers for TC.[24]

miRNA signature in TC

TC is divided into four main classes: PTC, FTC, poorly-differentiated carcinoma, and ATC. Differential expression of miRNAs is useful in the diagnosis of non-specific thyroid nodules.[24,63]

PTC

Several papers have studied the profiles of miRNA in PTC. Mir-146B, Mir-222, Mir-221, and Mir-181B are the most retained miRNAs. MiR-221 and miR-222 share similar seed series. The abnormal expression of these two miRNA genes has been documented in several cancers. Tumor condenser and p27 cell cycle regulator have in several cases been described as a direct target for miR-221 and miR-222. Moreover, many studies have reported a significant decrease in p27 regulation in PTC compared with that in normal thyroid tissue. Low expression of p27 in PTC due to excessive expression of miR-221 and miR-222 may explain the observed prevalence rate in PTC cells.[64]

Analysis of the expression of miRNA in the mutant BRAF V600E versus wild-type tumors was carried out, and the result showed a higher level of miR-221 in B-Raf proto-oncogene, serine/threonine kinase mutagenic specimens, characterized by violent behavior, advanced stage of the disease, extra-thyroid invasion, and the development of lymph node metastases. It has been proposed that BRAF V600E improves expression by activating the NFkB pathway.[65,66]

Mir-146B is one of the mostly observed miRNAs in PTC compared to normal tissue and its expression is positively correlated with tumor aggression. The presence of extending the tumor beyond the thyroid gland, analysis is also of predicted targets for miR-146b. Studies have shown that beta-retinoic acid (RARβ) receptors had an alleged binding site of MiR-146b in the 3'UTR region. Furthermore, the expression of RARβ mRNA in PTC was considerably lower than that in normal tissue. RARβ expression in several cancers is reduced and has shown a tumor-

suppressive role in several studies. A pilot study on a group of patients treated with retinoic acid and advanced PTC, RARβ ligand, found that 38% of the patients reported reduced tumor size. In addition, 26% of them reported improved absorption of radioiodine. These results indicated that miR-146b is involved in the initiation and development of TC by targeting RARβ. MiR-222, miR-221, and miR-146b were found to be over-expressed in aggressive PTC since their expression was correlated with lymph nodes, distant metastases, the danger of recurrence, and the presence of BRAF V600E mutation.[20,67]

Numerous reports have also found the overexpression of miR-181b in PTC as opposed to normal thyroid tissue. Study of the mechanism through which miR-181b controls cell transformation and development of cancer has shown that miR-181b binds specifically to the CYLD of 3'UTR and inhibits its expression. For many cancers, CYLD is downregulated and inhibits the NFkB pathway. CYLD also causes apoptosis in many human cancers and is under-expressed.[20,68-71]

FTC

FTC is distinguished by the activation of RAS mutations and rearrangement of PAX8/PPARγ. miRNA expression profiles are very limited in FTC. Moreover, the regulatory decreases were observed from mi-199a-5p and miR 144 in FTC, while miR-197 and miR-346 were overexpressed in FTC.[72,73] Out of all the microRNAs showing major up- or downregulation in FTC in vitro, only miR-199a-5p was studied. This miRNA targets CTGF, which leads to cell cycle progression inhibition. miR-146b and miR-221 have been found to be significantly upregulated in FTC compared with those in normal thyroid tissue, indicating that these two miRNAs are not PTC specific, but common to well-differentiated TCs.[20,74,75]

ATC

The most aggressive type of TC is ATC, which causes 14%–39% of the TC-associated deaths. ATC is resistant against most traditional therapies and refractory to the use of radioiodine.[5,76,77] ATC can be derived from a well-differentiated, pre-

existing cancer. Research has reported that the existence of mutation characteristics of differentiated TC, such as BRAF and RAS, as well as TP53 mutation, is specifically found in ATC. In ATC only a few miRNAs are dysregulated (miR-200a, b, and c). Multiple metastatic tumors were identified with the downregulation of miR-200 family members. The miR-200c is transcriptionally regulated by TP53 and the inactivation of TP53 mutations in ATC results in the downregulation of miR-200c.[78] miR-30 family members specifically distinguish between ATC and differentiated TC because miR-30 downregulation could be identified only in ATC; this indicates that miR-30 plays a role in the differentiation and development of TC.[79,80] The miRNA pattern in ATC implies that the family members of miR-30 and miR-200 are unique to undifferentiated thyroid tumors and involved in cancer development.[20]

PDTC

Research on miRNA profile in PDTC tumors has shown that miR-183-3p is upregulated in PDTC versus PTC and normal tissue. miR-150 and miR-23b have both been de-regulated in PDTC. These miRNAs were related to poor prognosis in many human cancers and are substantially correlated with tumor relapse and PDTC mortality.[20]

MTC

MTC is a tumor of the neuroendocrine arising from parafollicular (C) cells. miR-129-5p was shown to be significantly downregulated in MTC relative to normal tissue and overexpression of miR-129-5p in vitro decreased cell invasion and migration and inhibited AKT phosphorylation.[20,81,82]

Circulating miRNAs in TC

Recent studies have shown that miRNA can be found in blood, saliva, urine, and milk, indicating cell-based secretion of miRNA and possible cell-cell communication implications. Hence, circulating miRNA can be altered in specific pathological disorders, such as cancer.[83,84] Circulating miRNAs may be either secreted from dead cells via active secretion in exosomes or with RNA-binding proteins (RBPs), such as AGO2 or NPM1. miRNAs are protected from the degradation of the RNase either by active vesicle secretion or RBP binding.[85]

The expression profile of miRNA in tumor cells and exosomes is similar, which indicates that circulating miRNAs may be used as cancer diagnostic markers. miR-146b and miR-155 circulating in TC discriminated between BN lesions and PTC. miR-221 and miR-222 were found to be high in serum samples of PTC patients. Comparing the secretion of miRNA in the PTC serum to those with BN lesions showed that the circulating levels of miR-25-3p and once-miR-451 in PTC patients were substantially higher. Following surgical removal, both miRNAs were substantially reduced. Such data revealed that miRNAs are cancer cell secretions and can be used as non-invasive diagnostic markers.[20,86-88]

Using miRNA in combination

While FNA biopsy with the cytological examination is the most sensitive and precise diagnostic method for thyroid nodule diagnosis, in 10 to 30% of cases, it is not conclusive, which leads to indeterminate or suspicious diagnosis due to its low sensitivity despite its high specificity. Detection of BRAF V600E is currently used as a molecular test to enhance the detection of thyroid nodules, but it is not sensitive enough. It is because most malignant tumors do not have the BRAF V600E mutation. This led the researchers to conduct studies employing a combination of markers.[89]

For example, Italian researchers conducted a study on 118 samples of different patients using mRNA expression of four genes (KIT, TC1, miR-222, miR-146b) in order to identify novel computational molecular markers and enhance the differentiation between BN and malignant thyroid tumors. They obtained 118 pre-surgery thyroid FNA samples. The cytology cases involved in their study belonged to the patients with thyroidectomy examined based on normal histological guidelines, and all the participants had one FNA sample of the tumor. They only used cases with bonus slides for ethical purposes, and reference thyroid cells on the slides, chosen by professional cytopathologists, were utilized

for molecular study. By pyrosequencing and assessing the mRNA expression of the four genes (KIT, TC1, miR-222, miR-146b) via quantitative polymerase chain reaction (PCR), the collected 118 FNA samples were identified for the presence of the BRAF V600E mutation (exon15). They built computational models (Bayesian Neural Network Classifier, discriminant analysis), and tested their capability to distinguish between BN and malignant tumors.[8]

The results demonstrated in total that the V600E mutation was held by 36/70 malignant samples while all 48 BN samples were wild type BRAF exon15. The Bayesian neural network (BNN) and discriminant analysis, involving the expression of four mRNA genes (KIT, TC1, miR-222, miR-146b), presented a highly potent predictive value (94.12% and 92.16%, respectively) in the differentiation between malignant from BN tumors. The discriminant analysis revealed that 100 % of the samples in the malignant category were correctly identified and 95 % by BNN. Employing the four-gene model, they concluded that this method could be a step towards diagnosing suspicious tumors in patients with the indeterminate cytological examination and B-Raf proto-oncogene and serine/threonine kinase wild-type molecular markers, owing to its ability to specifically distinguish malignant from BN indeterminate thyroid FNA lesions using a panel of two miRNAs and two genes (miR-146b, miR-222, KIT, and TC1).[8]

Long non-coding RNAs (lncRNAs)

lncRNAs are molecules over 200 nucleotides long, which cannot encode protein products. lncRNAs play a crucial role in gene modulations.[63,90] They are involved in the regulation of various major cellular lncRNAs and can act as oncogenes or tumor suppressor genes. It has been stated that many lncRNAs are aberrantly expressed in TC and are able to act as biomarkers. Additionally, they have provided high sensitivity and specificity in the diagnosis of TC (genes involved in cell differentiation, proliferation, cell cycle, apoptosis, migration, and invasion primarily through gene expression modulation (Figure 3). The role of lncRNAs in cancers is that during tumorigenesis, lncRNAs can act as

oncogenes or tumor suppressor genes. It has been stated that many lncRNAs are aberrantly expressed in TC and can act as biomarkers. They have also provided high sensitivity and specificity in the diagnosis of TC.[90,91]

lncRNAs: Diagnostic markers in TC

lncRNAs are effective biomarkers owing to not coding for proteins and the fact that most of their functions are expression-related. The expression pattern of lncRNAs is tissue-specific and is greater than that of mRNAs, which allows them to be highly specific biomarkers for diagnosis. In several studies, lncRNAs have been reported to play functional roles in TC, including metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), PTC susceptibility candidate 3 (PTCSC3), Maternally Expressed Gene 3 (MEG3), LncRNA focally amplified lncRNA on chromosome 1 (FAL1), and nuclear enrichment expression abundant transcript 1 (NEAT1). An investigation in 2017 indicated that PTCSC3/miR-574-5p controlled Wnt/β-catenin activity and mediated PTC-1 cell proliferation and migration, which was essential for TC therapy and prognosis.[90,92]

It was revealed that high NEAT1 facilitated both the initiation and malignant progression of TC by controlling the expression of miRNA-214. Meanwhile, MALAT1 may act as both an oncogene and a tumor suppressor in various types of TC and play an important role in epithelial to mesenchymal transition in TC. These results indicated that lncRNAs act as TC biomarkers.[93]

The lncRNAs Biomarkers in TC
NEAT1

lncRNA was found to be closely associated with tumor progression of various malignancies, including PTC.[94-96] Li et. al showed that NEAT1 was upregulated in TC cells and that NEAT1 overexpression facilitated the growth and invasion of TC cells.[97]

FAL1

In TC tissues, FAL1 is substantially higher compared with that in matched normal thyroid tissues, and patients with elevated FAL1 expression are more likely to report multifocality compared to those with low FAL1 expression.[90,98]

MEG3

In TC, MEG3's ectopic expression inhibited cell migration and invasion by specifically inhibiting the expression of Rac1 at transcriptional levels by targeting its 30 UTR. Furthermore, MEG3 was substantially reduced in metastatic lesions in the lymph node compared with the associated non-metastatic lesions in TC.[99]

PTCSC3

PTCSC3 is in the immediate vicinity of SNP rs944289 in 14q13.3. It is known to induce PTC predisposition, the expression of PTCSC3 is exclusively thyroid-specific and substantially downregulated in PTCs relative to control subjects.[100]

MALAT1

MALAT1 is a lncRNA that participates in cancer progression control and is deregulated in different cancers. Several studies have shown that MALAT1 raised expression during progression from normal thyroid to PTC; however, it decreased in PDTC and ATC compared with that in normal thyroid tissue.[93] Early cancer detection improves the probability of survival significantly. These lncRNAs could be used as minimally invasive diagnostic and prognostic biomarkers for the evaluation of metastatic TC.

Immunohistochemical markers

Many authors have investigated the use of immunohistochemical markers and molecular profiling to help the diagnosis of thyroid follicular lesions. Even though the findings of these studies were controversial, some markers have shown clear results.[101] Numerous malignancy markers have been identified, all of which have certain benefits and limitations. GAL3, HBME-1 (Hector Battifora Mesothelial cell), and CK19 are amongst the most promising markers for thyroid pathology.[102]

GAL3

It is a beta-galactosyl-binding lectin involved in cell-cell interactions, apoptosis, cell growth regulation, and neoplastic transformation. GAL3 is an about 30-kDa protein found primarily in the cytoplasm, but also in the nucleus and on both the epithelial and immune cell surfaces. Studies have recorded changes in the expression of this lectin in the breast, gastric, and colon carcinoma during tumor development and metastasis.[103] It has been proposed that GAL3 may play a role in the malignant transformation of thyroid cells because of its role in cell transition and neoplasia. Some research has shown that positive expression of GAL3 is specifically for the malignant thyroid FDLT, where many previous studies on thyroid tissue have found the expression of GAL3 to be a marker of malignant, but no expression of GAL3 in BN or normal cells.[101,103,104] GAL3 has been identified to be a very sensitive and credible diagnostic marker for pre-operative diagnosis of thyroid tumors with high sensitivity and specificity in cytological cell blocks, tissue samples, and in fresh cytological samples. This marker was initially intended for malignancy measurement and as a possible tool for the differential diagnosis of follicular thyroid lesions.[102]

HBME-1

HBME-1 is a monoclonal antibody developed by mesothelial cells against an unknown membrane antigen. It was initially found in malignant mesothelioma; multiple studies have shown that HBME-1 plays a significant role in PTC diagnostics.[104] However, with Hürthle cell and apocrine-like changes, the expression of HBME-1 tends to decrease.[102]

TPO

TPO is a membrane enzyme concerned with thyroid hormone synthesis. It has a key role in the metabolism of iodine, being important for the function of the thyroid.[104] In contrast, it has been identified to be used in follicular adenomas (FAs) diagnosis. It represents normal thyroid activity and should therefore not be expressed in malignant cells whatever is their histopathologic state (papillary, medullary, follicular, or anaplastic).[105,106] Some articles have been conducted showing the significance of TPO in the diagnosis of thyroid nodules. Even in cases where the specificities varied from 68 to 90%, the sensitivities were consistently excellent between 97% and 100%.[106] More than 95% of papillary and follicular thyroid tumors have rapidly changed antigenically. This condition is developed

early in follicular carcinogenesis and is associated with tumor growth and cell atypia. TPO immuno-histochemistry (IHC) with monoclonal antibody 47 (mab 47) can be utilized to detect TPO on thyroid FNA samples.[107]

CK19

A third marker, CK19, has a low molecular weight compared with other cytokeratins, which is widely present in simple epithelial cells and basal cell layers of stratified epithelium.[104] It may be valuable for the detection of papillary carcinoma (PC) as it has exhibited a high diffuse cytoplasmic reactivity.[102] The expression of CK19 in thyroid neoplasia is generally limited to papillary carcinomas. If this is proven true, antibodies to CK19 may be used to differentiate the papillary carcinoma follicular type from follicular lesions and nodular hyperplasia.[103]

Among recent studies of new immunohisto-chemical markers, GAL-1 has shown higher specificity (97%) than GAL3 and CK19, which indicates higher sensitivity (97% and 98%, respectively), and therefore, complementary diagnostic values. Additionally, Ki67 was also used as an effective single marker for the differentiation between carcinomas and BN TCs. Marker combinations were investigated to enhance the differential diagnosis regarding the identification of follicular thyroid tumors. The correlation between GAL3, CK19, and HBME-1 is the most significant method to distinct between PTC and follicular adenoma (FA); 97% precision, 95% sensitivity.[23,101,108,109]

Additional new combinations of markers have opened new doors to the diagnosis of TCs. Combined positive emerin (including improvements in the nuclear level) and negative CD56 (lost in cancer) have reported 72% sensitivity and 100% specificity and could be useful as additional markers in similar cases with high diagnostic validation (high specificity and PPV). Similarly, quantitative PCR and IHC examined the differential expression of trophoblast cell surface antigen 2 (TROP-2) and stomatin-like protein-2 in PTC and FA, and findings confirmed that marker expressions in carcinomas were substantially increased. Moreover, when TROP-2 was combined with CD56, the sensitivity and NPV increased to 100% and had a higher diagnostic accuracy. Lately, neuregulin 1 was reported to be significantly expressed in PTC compared to the neighboring normal tissues.[23]

Concerning the inability to accurately differentiate between BN and malignant nodules, a study was conducted by Arcolia et al. using a combination of GAL3, CK19, and HBME-1 immunostaining to improve the reliability of TC diagnosis.[110] Two tissue microarrays, consisting of 66 FAs and 66 papillary carcinomas (PC), were diagnosed with TC IHC for CK19, galectins [galectin-1 (GAL-1), GAL3 (GAL-3), galectin-7 (GAL-7), and galectin-8 (GAL-8)], Hector Battifora Mesothelial Epitope-1 (HBME-1), and TPO.[105,110]

They examined the diagnostic efficacy of galectin-1 separately and in combination with four other TC markers used in medical practice. The performance of individual or combined immuno-markers in TC diagnosis and the evaluation of the optimum cut-off points for malignancy diagnosis was measured using the receiver operating characteristic (ROC) curves and the area under the ROC curve (AUC) assessment. Based on the cut-off points, the precision, sensitivity, PPV, and NPV of individual and combined markers were evaluated from crosstabs, and the significance was determined via the Fisher's exact test. P-value < 0.05 was considered to be significant.[110]

The study results revealed that GAL1, GAL3, CK19, and HBME-1 signal intensities were significantly higher in PC relative to those in FA ($P < 0.001$). Conversely, TPO expression levels increased significantly in FA compared with those in PC ($P < 0.001$). The most sensitive markers were confirmed to be GAL3 and CK19 (97% and 98%, respectively) while GAL-1, as a single marker, appeared to be the most specific one (97%). Their results also showed that various immunomarkers profiles have non-redundant distribution, suggesting a non-overlapping functional spectrum. Galectins are spread widely in a tissue- and cell-specific way. This is especially noted for GAL-7 and Gal-8, which are not

expressed differently between FA and PC, indicating that these galectins are not engaged in thyroid tumor growth or that their expression has not been changed by the transformation of tumor cells.[110]

They also identified that GAL-1, GAL-3, and CK19 can be used in combination in order to improve the differentiation between malignant and BN thyroid tumors. Therefore, when two to five markers are combined, the specificity, sensitivity, and the PPVs and NPVs for malignancy are remarkably enhanced. The combination of CK19, GAL-3, and HBME-1 was the most effective and sensitive marker panel with the highest specificity and sensitivity (97% and 95%, respectively) for differentiation between PCs and FAs. This supports that the combination of such markers can enhance the accuracy of TC diagnosis.[110]

There are also two studies, one of which was conducted by Barroeta et al., 2006,[101] and the other one by De Matos et al., 2005.[102] Both studies' results are similar to Arcolia et al. study,[110] to enhance TC diagnosis by combining immunomarkers. Both resulted in the high specificity of GAL-1 as a single marker. They concluded that the use of this combination enhances the diagnostic accuracy in differentiating malignant and BN tumors.

Until recently, it has been thought that clinical and histopathological factors are much more important in TC diagnosis and risk stratification is more important than genetic ones. However, each subsequent publication on TC molecular aspects has provided evidence on the significance of genetic alterations not only in the tumorigenesis, but also in TC aggressiveness. Recent studies have indicated that some genetic alterations might be used as markers of TCs aggressiveness.[111] However, the most important molecular markers (signaling pathway) of TCs can be summarized as the following: Rare B-Raf proto-oncogene and serine/threonine kinase (BRAF) alterations [like K601E mutation; MAPK signaling] are involved mainly in follicular variant of PTC (FVPTC) and papillary microcarcinoma (PTMC). Meanwhile, KRAS mutations (MAPK signaling) are in

FVPTCs with favorable clinical and sonographically profiles (indolent TCs). Different molecular markers are also involved in aggressive follicular cell-derived TCs PTC, ATC, PDTC, FTC. BRAF V600E mutation (MAPK signaling pathway) is a molecular marker for PTCs. TERT promoter mutations [C228T (MAPK signaling) and C250T (WNT signaling)] are markers for ATC, PDTC, FTC, and PTC. These TERT promoter mutations in most PTC cases coexist with BRAF V600E mutation. These TERT promoter mutations also occur in various types of TCs according to the following frequency: ATC > PDTC > FTC > PTC, being the highest in ATC. TP53 inactivating mutations (TP53 signaling) are mainly in ATCs and PDTCs and also present in a small fraction of aggressive PTCs and FTCs; CTNNB1, APC, and AXIN1 mutations (WNT signaling) are mainly in ATC, and PTEN, AKT1, and PIK3CA mutations (PI3K/AKT signaling) in advanced TCs, mostly in ATCs. miR-146b in aggressive BRAF-positive PTCs and RAS mutations (MAPK signaling) are present in a high fraction of ATCs and PDTCs, but also in FTCs. RAS mutations have a controversial role as a molecular marker of aggressive TCs because of their presence in follicular adenomas (FAs) and papillary-like nuclear features (NIFTP). RET/PTC rearrangements (MAPK pathway) are involved in other follicular cell-derived TCs (a molecular hallmark of radiation-induced PTCs).[111]

Ancillary markers and studies of TC

As mentioned, it is quite difficult to distinguish between BN and malignant thyroid lesions as the diagnosis of thyroid tumors is based on histologic features, especially in lesions with a follicular pattern. Thus, extra studies, such as IHC, which was mentioned earlier, may be advantageous. Various markers have been evaluated, including two IHC markers (TROP-2 and HBME-1) in the diagnosis of TCs. IHC panel consisting of trophoblastic cell surface antigen-2 (TROP-2) and HBME-1 can be used in equivocal follicular patterned lesions for the diagnosis of thyroid carcinomas. Overexpression of TROP-2 in carcinoma is related to poor prognosis and aggressive behavior. No single marker is sensitive

or specific for the differentiation between BN and malignant thyroid neoplasm, but the combination of TROP-2 and HBME-1 use can accurately diagnose carcinoma with equivocal morphology with high sensitivity and specificity.[112] FNA cytology, as discussed previously, is the key procedure to diagnose thyroid nodules and does not provide definitive results in some groups of patients. The use of molecular markers testing has been described as a useful aid in the differentiation of thyroid nodules, which presents with an indeterminate cytodiagnosis. The Afirma GEC, mutational assay, and immunohistochemical markers, have been all widely used to further enhance the accuracy and postpone unnecessary surgeries for BN thyroid nodules. Nevertheless, their synergistic uses can predict the risk of malignancy and give an accurate diagnosis. However, when both the routine gold-standard FNA cytology and molecular testing are used together for a diagnosis, the diagnostic accuracy will increase for indeterminate thyroid nodules. As shown by different studies, these helpful tests are highly advantageous when more than one or all the biomarkers are used instead of a single marker for pre-surgical diagnosis. The difficulty in the distinctness of follicular neoplasia, Hurthle-cell neoplasia, and the follicular variant of PTC makes it important to merge these tests into clinical practice. To reduce the number of false results of negative FNA cytology and subsequent diagnostic surgeries, molecular testing bears are promising for the management of patients with thyroid nodules suspicious of malignancy.[113]

The indications for additional studies on fine needle aspirate of the thyroid are based largely upon the cytomorphologic features of the FNA sample. Most of these extra studies depend on the identification of proteins particularly associated with various lesions and aim at the characterization of suspected malignancies involving the thyroid, specifically medullary carcinoma, anaplastic carcinoma, and lymphoma, or a metastatic carcinoma to the thyroid. FNA can be also utilized when there is a suspicion of a parathyroid lesion instead of a thyroid process and suspected cases

of metastatic thyroid carcinoma to the lymph node. A more debatable area involves the utilization of ancillary studies to reclassify an indeterminate/suspicious FNA into a BN or malignant category or to refine the risk of malignancy within this category. In addition, the clinical setting should be considered for making the decision about doing ancillary studies, specifically a family history of TC, a history of other cancer, or a rapidly growing firm nodule. Different ancillary studies have been widely employed in the detection of specific proteins using different immunologic techniques, including IHC on cell block preparations. IHC is commonly used for the characterization of suspected thyroid malignancies; pre-analytical sampling and processing protocols and variables are very important in the elucidation of the results from an ancillary study of the relatively limited cellularity of thyroid FNAs, which raises challenges for any ancillary study. Furthermore, the material obtained for cytomorphologic analysis should not be compromised by the ancillary study.[114] The ancillary study and sample preparation depends upon the suspected carcinoma as mentioned. However, the ancillary studies in thyroid FNAs and sample preparation can be summarized as the following: in the case of suspected medullary carcinoma, IHC panel (calcitonin, thyroglobulin, carcinoembryonic antigen (CEA), chromogranin), and serum calcitonin is used on cell block (CB) from FNA, preferably including at least one dedicated pass is prepared. This preparation can also be used in suspected anaplastic carcinoma with IHC for pan-cytokeratin. Moreover, flow cytometric immunophenotyping ancillary study using live cells in a supportive medium, preferably including at least one dedicated pass, is used when the indication is suspected to be lymphoma. In suspected metastatic carcinoma, IHC for thyroid transcription factor-1 (TTF-1), expand panel is preformed to identify primary malignancies, if TTF-1 is negative and on CB from FNA, preferably including at least one dedicated pass is performed. In the case of suspected parathyroid tissue, the ancillary study is either IHC for TTF-

1, parathyroid hormone (PTH), and chromogranin or may also consider PTH level on FNA sample; sample preparation: CB from FNA, preferably including at least one dedicated pass is prepared. When suspected metastatic thyroid carcinoma to lymph node, the ancillary study is either IHC for TTF-1, thyroglobulin, and calcitonin or may also consider thyroglobulin level on FNA sample; sample preparation: CB from FNA, preferably including at least one dedicated pass is prepared. About indeterminate/ suspicious FNA, there is insufficient evidence for either IHC or molecular techniques.[114]

c-KIT or CD117 is a transmembrane tyrosine kinase receptor for the stem cell factor (SCF) encoded by the c-KIT proto-oncogene. c-KIT/CD117 downregulation has been described as an ancillary marker for PTC in FNAB. Differences have been found concerning immunoreactivity for CD117 between PTC and BN thyroid nodules (BTNs). Therefore, it has been suggested that CD117 is expressed in both normal follicular epithelium and BTNs. In contrast, the expression of CD117 is absent or weak in PTC. These results suggested that CD117 may be useful as an ancillary marker for PTC. Thus, it has been demonstrated that CD117 immunoexpression in classic PTC is decreased significantly compared with the expression in normal follicular epithelium and BTN and that this feature can be detected immunocytochemically in thyroid FNAB specimens. These findings suggested the possible role of CD117 as an ancillary marker in the evaluation of thyroid FNAB specimens.[115,116]

Conclusion

In summary, in cytologically indeterminate samples, molecular FNAB tests were reported to enhance the diagnostic reliability of the thyroid nodules. Critical developments in studying the molecular pathways that cause carcinogenesis of the thyroid have resulted in the advancement of the patients' diagnosis. Nowadays, new marker panels and signatures are often in the process of being validated to establish additional enhancement for the distinguishing thyroid nodules. The combination of new markers, such as immunohistochemical protein identification, genetic modification (mutation and rearrangement), and miRNA (up / downregulation) levels, have been evaluated and validated in a wide range of tissues to provide the maximum sensitivity and specificity to significantly enhance the accuracy of TC differential diagnosis.

Conflict of Interest

None declared.

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