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REVIEW ARTICLE

Molecular testing approaches in thyroid cancer diagnosis

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ABSTRACT

Thyroid cancer (TC) is the most common endocrine malignancy worldwide, with an annual incidence of around 300,000 cases. In recent decades, the incidence of TC in many countries, including Saudi Arabia, has increased significantly. Genomics has allowed multiple mutations to be examined simultaneously across multiple genes, providing a detailed genomic profile for a given tumor. It is understood from a molecular perspective that different signaling pathways may have genetic abnormalities as the primary factors in thyroid tumorigenesis. While there is still early progress in the usage and standardization of targeted next-generation sequencing (NGS) in TC, a major ongoing study to distinguish malignant from benign thyroid nodules from fine-needle aspiration (FNA) has shown promising results that can prevent unnecessary surgery, based on NGS analyses of mutations and gene fusions associated with most TC. The differential diagnosis and malignancy risk stratification of TC require multidisciplinary skills and experience of both ultrasound and the FNA and molecular analysis. The most common TC mutations are point mutations in the *BRAF* and the chromosome rearrangements of *RET*/papillary TCs. The transition mechanisms tend to be linked to specific etiological features, which are very crucial while deciding the treatment protocol. On the other hand, FNA cytology has an intrinsic drawback. The findings of infinite cytology cannot differentiate some types of TC, such as follicular adenomas, thyroid follicular carcinomas, or papillary thyroid follicular. Nevertheless, accumulating evidence indicates that molecular diagnostic approaches can overcome these limitations. In this review, we present an updated summary, which focuses primarily on molecular alterations in the tumorigenesis and biomarker investigations of TC.

Keywords: Thyroid cancer, molecular testing, gene panel, NGS, FNA, BRAF.

Introduction

Cancer of the thyroid gland

Cancer of the thyroid gland or thyroid cancer (TC), in short, grows through the thyroid tissue (1). TC is a common endocrine-based malignancy with a steadily increasing incidence. Recent advances in understanding its molecular pathogenesis are mainly explained by the fundamental function of several primary signaling pathways and associated molecular distributors (2). Thyroid tumor cells are derived from epithelial thyroid cells, and it had less manifest early clinical symptoms (3). The major four types are papillary TC (PTC), follicular TC (FTC), medullary TC, and anaplastic TC (ATC) (1). TC prognosis depends on the type of cancer and the stage at the diagnosis. The overall prognosis is excellent for the most common type of TC, such as PTC (1). In recent years, the increased incidence of thyroid carcinoma has possibly been related to the rising and preceding diagnosis. There is currently no consensus on the benefits of this trend toward earlier diagnosis (4). Thus, ultrasound (US) combined with fine-needle aspiration (FNA) is considered a relatively high diagnostic value to distinguish between benign and malignant thyroid lesions in suspicious thyroid nodules (TNs) and can be used as a preferred approach for the clinical management (2). However, based on the Bethesda System for Reporting Thyroid Cytopathology (20%-30%) of cytopathology, the results from thyroid FNA fail to detect malignancy or highly suspect (aka indeterminate) TNs (5). Consequently, it is clinically essential to use molecular genetic biomarker analysis in conjunction with FNA to improve the biopsies' diagnostic accuracy and the clinical decision-making process as they are now more easily accessible and

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thoroughly assessed. This review article presents an updated summary, which focuses primarily on molecular alterations in the tumorigenesis and biomarker investigations of TC.

Epidemiology

TC is a widespread head-and-neck malignancy, which has a high incidence rate of 56 cases per 100,000 people (6). Recently, TC ranked as the first growing neoplasm in systemic endocrine tumors (2). Over recent years, the prevalence of TCs has increased, posing a significant threat to human health. (1). As of 2015, 3.2 million people were affected by TC worldwide, most commonly from ages 35 to 65 years. Furthermore, women have been affected more frequently than men (2). TC became women's fastest-growing cancer in some populations worldwide, such as Saudi Arabia (7). Based on the last Saudi Cancer Registry published in 2019 by the Saudi Health Council (Supplementary material Appendix 1), TC incidence increased exponentially in the past two decades. This dramatic change in incidence made TC rank the second most common cancer in Saudi women (Figure 1). The increased occurrence of TC in Saudi Arabia is remarkably high compared to other nations as in those in the USA, as TC constitutes only 2.9% of all cancer cases and 4.6% of all female malignancies, making it the fifth most common cancer among females in the USA (8) > NOT (7,10). In Saudi Arabia, the reason behind the elevated prevalence of the TC incidence is attributed mostly to the massive improvement of the detection techniques, and as healthcare facilities became more accessible and affordable, the question of its prevalence in Saudi women is hitherto unknown. A histopathological study performed by Fazal Hussain et al. (7) on a total of 2,292 patients with TC, who were treated at King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia, from 2000 to 2010 revealed that 72% are PTC and 13.6% are FTC, whereas the other forms account for about 14%.

Molecular Alterations in Thyroid Neoplasms

A deep understanding of TC molecular tumorigenesis is mainly through the description of the role of several disruptions in key signaling pathways: primarily driver mutations that are responsible for tumor initiation and progression. The main cellular mechanisms in TC are the genomic changes such as deoxyribonucleic acid (DNA) mutation and chromosomal rearrangements or gene fusions, which can occur due to chromosomal inversions, deletions, or translocations in the genome (9). The most common genetic alteration in TC is the activating mutation in the gene for the B-type Raf kinase, *BRAF* (Figure 2) (10). *BRAF* is located on chromosome 7 and is the most potent activator of the mitogen-activating kinase pathway among Raf kinases. BRAF-activating mutations are restricted to the kinase domain of exons 11 and 15 (10). The $BRAF^{V600E}$ mutation at nucleotide position 1799 transforms thymine into adenine, resulting in a conversion of valine to glutamate. The $BRAF^{V600E}$ mutation was observed in up to 80% of TCs. It is most frequently found in PTC and poorly differentiated TC (DTC) and ATC that coexist with or arise from PTC (11). This gene encodes a protein belonging to the RAF family of serine/threonine protein kinases (11). It is a potent activator of the mitogen-activated protein kinase (MAPK) signaling pathways. BRAF is downstream of *RAS* and *RET* signal pathways (11).

BRAF mutations are identified in a wide range of human cancers, with the highest prevalence in melanoma and TC (12). $BRAF^{V600E}$ mutation strongly enhances *BRAF's*

Figure 1. *The prevalence of the most common cancers in Saudi Arabia shows thyroid cancer in > is the second most common cancer in Saudi Arabia Women. (Data were extracted from the Saudi Cancer Registry Report in 2019).*

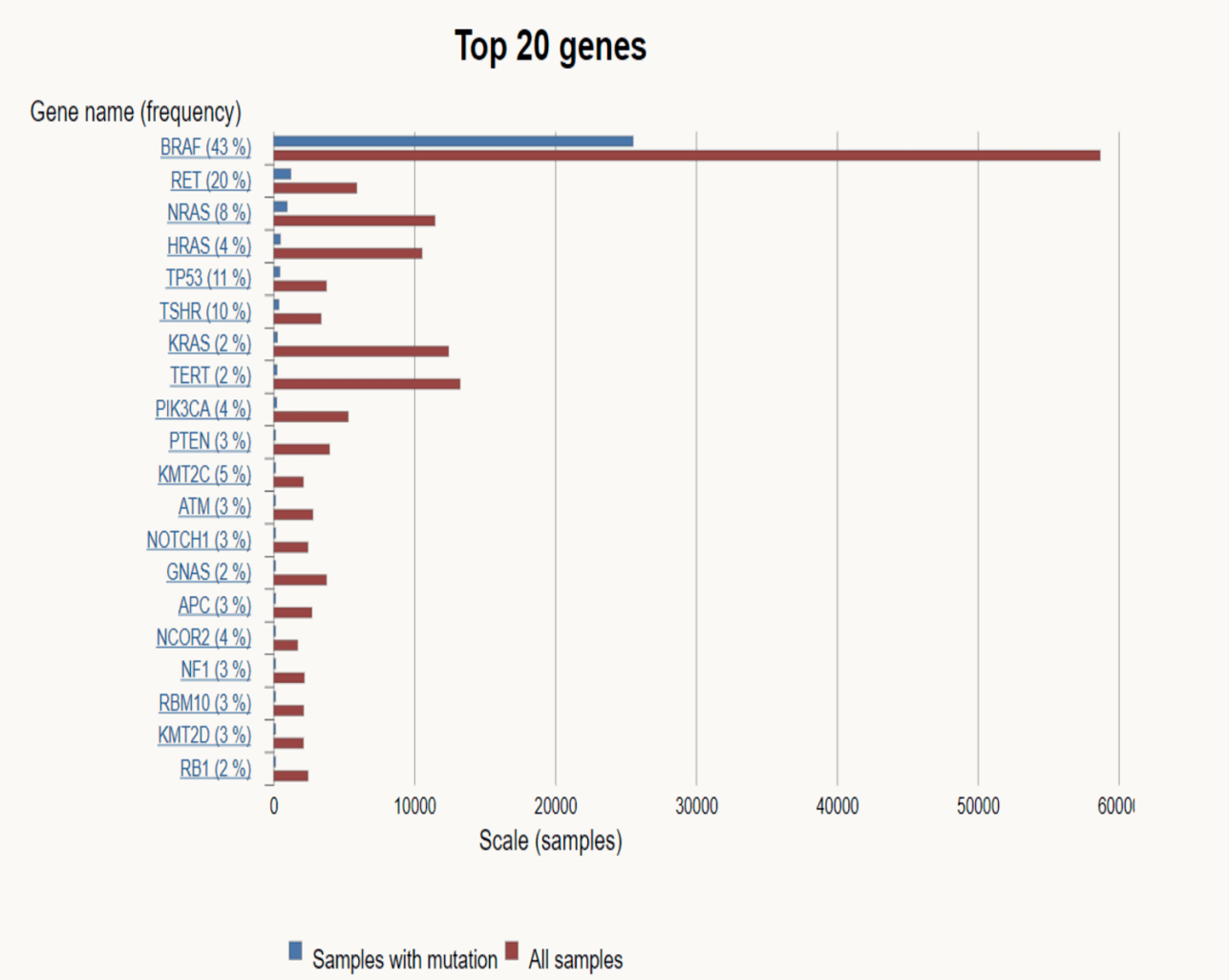

Figure 2. *The top 20 mutated genes in thyroid cancer (Source: COSMIC 2020).*

kinase activity with a 480-fold increase in Extracellular-signal-Regulated Kinase (ERK) 1/2 phosphorylation compared to wild *BRAF*, which triggers a certain mutation in cellular proliferation, differentiation, tumorigenesis, and promotion of epithelial-mesenchymal transition (13). The activation of MAPK and PI3K-AKT signaling pathways is essential for TC initiation and progression (Figure 3) (11). The *BRAF*-mutated PTC displays an inadequate response to radioiodine therapy with high-frequency lymph node metastases and relapse after thyroidectomy (11). Their refractoriness to radioiodine appears to be due to the high MAPK-pathway output driven by the $BRAF^{V600E}$ oncoprotein that suppresses gene expression required for iodide incorporation. The irregular stimulation of the MAPK cascade due to mutations and rearrangements in the *BRAF, RET,* and *RAS* genes characterizes approximately 70% of the PTC cases. Among these, the reordering of RET genes leading to the formation of a constitutively active fusion protein occurs in 10%-20 % of the cases (14).

Both *in vitro s*tudies and transgenic models of BRAF suggest that $BRAF^{V600E}$ mutation promotes progression to TC and is linked to the invasion TC phenotype. Based on these findings, several studies showed an association with an aggressive tumor phenotype with the presence of a $BRAF^{V600E}$ mutation in TC (15). Some studies indicate that $BRAF^{V600E}$ mutation is associated with low prognostic factors including the older age, male sex, extrathyroid invasion, lymph node and distant metastasis, higher stage of the tumor, and even higher levels of chronic disease (16).

The proto-oncogene rearranged in neural crest origin cells during transfection (RET) encodes a membrane tyrosine-kinase receptor. Thyroid follicular cells may also express the domain of RET tyrosine kinase. Somatic RET translocation has been identified in some sporadic and radiation-induced PTCs. PTC contains RET/PTC chromosome rearrangement. In this gene rearrangement, a fraction of the RET gene is fused to one of the several genes, and RET/PTC1 and RET/PTC3 are the most common rearrangement types, in which RET is fused to. The RET tyrosine kinase domain, when constitutively activated due to RET rearrangement, can lead to PTC. About 10%-20% of sporadic PTC contain RET/PTC rearrangements. Patients with a history of

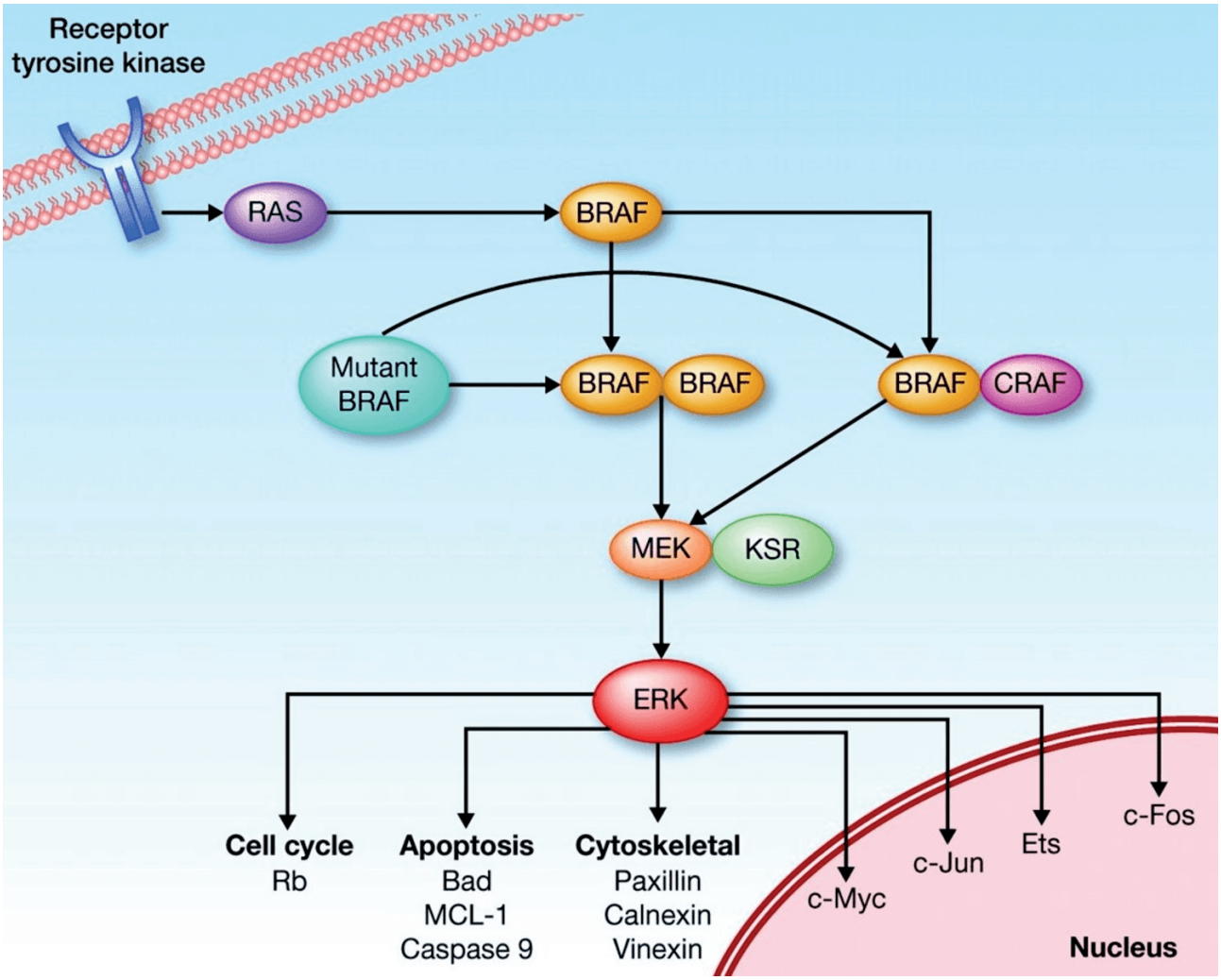

Figure 3. *Typically, the MAPK-signaling pathway is activated by the activation of a tyrosine kinase receptor, which activates RAS, which in turn promotes homo- or heterodimerization of wild BRAF. The activated BRAF MEK (KSR-bundled) phosphorylates ERK leading to several cellular effects such as proliferation and survival. Without RAS activation, mutant BRAF can dimerize and activate MEK. (Adapted from: Role of BRAF in thyroid oncogenesis by Lisa M. Caronia et al. 2011, Clinical Cancer Research) [14].*

radiation exposure PTC in children and young adults have a higher frequency of RET/PTC rearrangements (17). The RET gene is located near the centromere on chromosome 10q11.2 and contains 21 exons. In 1985, Takahashi et al. (18) first described that *RET* (rearranged during transfection) is a proto-oncogene, which may be triggered by cytogenic rearrangement. After 3 years, the same investigators cloned the *RET* gene. This gene codes a plasma membrane-bound tyrosine kinase enzyme, the RET receptor. The RET gene is expressed by neuroendocrine thyroid C cells, adrenal medullary cells, and parathyroid cells (17).

The tumor suppressor gene *p53* mutations are believed to occur in late thyroid carcinogenesis with a higher incidence of PTC and, ultimately, ATC. Understanding the existence of a *p53* mutation, while not currently targetable, is an informative mechanism for resistance and might be considered as a potential biomarker that promotes a more aggressive clinical course (19).

The germline variants in chromosomes (9q22.33 and 14q13.3) are associated with a high risk of thyroid differentiation (20). These loci contain two main genes such as *FOXE1* and *NKX2-1*, the main thyroid regulators, and differentiated function. A minimum of 3%-9% of DTC is familial. In general, these thyroid carcinomas occur as an individual family entity identified as first-degree relatives of the disease (20).

Molecular Diagnosis of Thyroid FNA

Considering that many TC tumors are indolent tumors, and many patients may have an excellent prognosis even though their diagnosis and treatment have been delayed (21). While TC usually has a good prognosis, nearly 10%-15% of TC patients have recurrences. Approximately 5% will develop a metastatic disease that is not responsive to radioactive iodine (RAI) and, ultimately, will die from this disease. Of these purposes, patients with suspicious TNs require an early diagnosis of TC.

Together with FNA cytology (FNAC), US is determinant in the classification of malignant TNs. To improve FNAC accuracy in detecting malignancies, testing for oncogene mutations has been proposed (14,15), suggesting that it enhances FNA diagnosis performance, and when it is performed in indeterminate cytopathology, it permits to obtain the diagnosis in many cases. Testing for multiple mutations (BRAF, RAS, and RET/PTC) improves the performance and increases the specificity and sensitivity (22). Nonetheless, in situations under which FNA has conducted patients, healthcare providers face indeterminate cytology. It was even less apparent from the recommendations of how the same principles can be used to make management decisions. This condition has made it possible to strengthen the cytological inaccuracy inherent in the indeterminate thyroid FNA diagnosis (5).

An increased analysis of genetic lesions acquired that the difference between carcinoma and benign nodules has significantly extended the understanding of DTC molecular pathogenesis. In the DTC, several different molecular biomarkers have been identified and validated to help the diagnosis distinguish malignant TC (23).

A Comprehensive Approach for Genomic Profiling in TC

The next-generation sequencing (NGS) tests were used to evaluate TNs in indeterminate cytology. Moreover, FNA as a useful tool for assessing the TNs has some drawbacks as 20%-30% of FNA samples fall into the uncertain cytology category (5). Comparing with the FNA method, NGS methods allow simultaneous testing of proto-oncogenic activation in multiple signal pathways and tumor suppressor mutations (i.e., more in-depth and accurate diagnosis). Technological advances in NGS approach allow for the widespread screening of multiple genetic loci by using small input amount of the DNA in nanoquantities of tumor DNA even in very difficult clinical samples such as samples from formalin-fixed paraffin-embedded (24,21). Mutational profiling of TC will improve the early detection of TC and targeted therapy considerations afterward. NGS technology provides a molecular analysis of hundreds of genes of interest using targeted sequence panels. Recently, NGS-based molecular studies tend to be emerging rapidly for oncology study and clinical practice. Gene panels have traditionally been used to determine the genetic cancer predisposition by testing the variants of several susceptibility genes. In recent years, these panels have been used for testing the malignancy of solid tumors to evaluate somatic changes (25). Molecular testing of thyroid fine needle aspiration specimens was established with the primary goals of overcoming the ambiguity of indeterminate FNA cytology and preventing the commonly prescribed diagnostic surgery for TC patients. A Molecular testing of thyroid FNA specimens was established with the primary goals of overcoming the ambiguity of indeterminate cytology and preventing the commonly prescribed diagnostic surgery for these patients. A double-blind multicenter study published the results of clinical validation of the ThyroSeq genomic classifier in thyroid nodules with an indeterminate FNA cytology. For example, targeted gene panels may help where there is a suspicion in the diagnosis or non-conclusively malignant or benign in TN samples.

ThyroSeq® test

The initial studies include identifying single genome changes, followed by various targeted gene panels (26). Molecular testing for specific TC mutations is one way to enhance cancer detection sensitivity in individuals with cytologically suspicious TN (benign or malignant). There is currently a subset of identified mutation markers that can give a accurate result related to detection of genetic variations in TC, these markers panel developed by Nikiforova et al. (24) and it is called ThyroSeq® which stands for "Thyroid Sequencing". The sensitivity of ThyroSeq® test panel was first verified with DNA samples of known or no mutations in TC. DNA was also analyzed from 228 thyroid tissues, both neoplastic and non-neoplastic (26). Molecular profiles of various types of TC were examined, and point mutations were found in 30%-83% of specific types of cancer. The ThyroSeq® test had a 100% analytic accuracy, as it correctly detected all pathogenic mutations in previously positive thyroid tumor samples and cell lines. All mutations identified by ThyroSeq® were further confirmed by other molecular testing methods, such as real-time PCR and Sanger sequencing.

After it is successful lunching The ThyroSeq® and introduction into routine clinical practice in 2007 the genes in the panel increased from 6 genes with 65% sensitivity in (TheroSeq v.0) to 112 genes with 94% sensitivity in (ThyroSeq v.3). The final version of the ThyroSeq® can interrogate five classes of genetic alterations: (i) point mutations (SNVs), (ii) insertions and deletions, (iii) gene fusions, (iv) gene expression alterations, and (v) copy number alterations (CNAs) (26).

The findings of ThyroSeq® help to assess the suspicious TN and to customized the treatment course (if needed). Negative ThyroSeq® findings will help to avoid surgical unnecessary thyroid excision in certain circumstances. If the test is positive, ThyroSeq® offers additional knowledge that lets clinicians to choose the most suitable procedure to deal with each case individually.

Limitations of Using Molecular Diagnosis in TC

The main drawback is that these molecular tests are not widely available, and they are relatively expensive. Moreover, they have a modest predictive value in some cases, meaning that several nodules are not malignant even the test is positive. On the molecular level, we know that $BRAF^{V600E}$ is an indicator of aggressive tumor behavior, particularly in PTC. Therefore, BRAF-positive tumor cells are more likely to be metastatic in regional lymph nodes. A high prevalence (30%-80%) in PTC constitutes an argument against the application of *BRAF* in surgical decision-making. However, it is anticipated to

help stratify diagnostic approaches to TC by gaining more experience and doing more molecular testing, including *BRAF*. For instance, thyroidectomy and node dissection may be required for BRAF-positive tumors.

Conclusion

The rapidly developing NGS technology offers a cost-effective solution to cancer genomics. The traditional categorization of cancers based on their histopathological appearance will be replaced by genomic profiling that identifies the unique molecular signature that precisely describes every tumor and may help personalized design treatment. NGS can detect tumor-specific genetic changes that can be used in patient monitoring and follow-up. Molecular testing of thyroid FNA specimens has been developed with the primary objective of addressing the mystery of indeterminate cytology and eliminating the diagnostic surgery typically prescribed for such patients. With the advent of NGS technology and the rapid discovery of TC markers, molecular testing for indeterminate TNs has been implemented. Genetic changes accumulated in tumor cells during TC progression induce both tumor growth and tumor progression consistently. $BRAF^{V600E}$ is the most common and earliest genetic event in TC, and it seems to be a good candidate gene for TC diagnosis and monitoring. Furthermore, new forms of mutation other than the primary tumor may be identified in the NGS examination of either circulating tumor cells or cell-free plasma DNA during RAI and TC treatment. The gene panel can also be applied during the clinical course to detect genetic changes linked to acquired resistance to treatment. Currently, the studies of TC genetics by NGS appear to focus on identifying genetic differences in different forms of TC. A novel approach to system biology in TC will also help to discover the association between different pathways, providing a new field for TC pathogenicity. Definitively, recent advancements in molecular diagnosis will contribute to significant advancement in the early detection and then better treatment for TC patients. Molecular thyroid tests aim to precisely determine the existence of thyroid malignancy and reduce cytologically indeterminate diagnostic ambiguity, which is highly suspicious TN before surgery. We think that it could be an additive to the current clinical risk stratification system when making operative or medical management decisions about the clinical relevance of the test of BRAF mutation status. This is also a pressing issue primarily because of the prevalence of small localized PTC, with the rise in TC incidence. We do not doubt that these advancements will continue to produce more effective therapies focused on more precise oncogenic targets to overcome treatment resistance.

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