

Roshan Lall Gupta's
Recent Advances in
SURGERY

Jaypee Brothers

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SURGERY

Vol. 14

Editor

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The Health Sciences Publisher

New Delhi | London | Philadelphia | Panama



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Roshan Lall Gupta's—Recent Advances in Surgery (Vol. 14)

First Edition: 2016

ISBN: 978-93-85891-18-2

Printed at:

Dedicated to

*My wife Ritu Ragini
Son Akshat and daughter Aanya*

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Roshan Lall Gupta's—Recent Advances in Surgery (Vol. 14) is focused for postgraduate and young practicing surgeons and will update them with latest developments in the field of imaging, planning and surgical techniques. In continuing the legacy of the previous editors, I have tried my best to cover all fields of surgery. It will help young surgeons to change their practice and will help postgraduate students to pass their examinations.

In this volume, the contributors are the eminent surgeons with good knowledge in their respective fields and they have delivered best of their knowledge with evidence of clinical practice. In present era, evidence-based practice has taken precedence and is to be strictly followed. In this fast-changing world, it is also imperative to keep abreast with the latest developments in the field of imaging, evaluation and management of the surgical problems. In surgical practice, it is important to have good knowledge of newer imaging modality so that it not only gives accurate diagnosis but can also help in planning surgery. With the use of internet, now patients too are aware of the latest treatment modalities available. The surgeons should also know in detail about those upcoming procedures with their limitations and complications.

I thank all authors for their contribution for this edition. They have made the text simpler and student-friendly so that it can be easy for young surgeons to understand and adapt in clinical practice. I am quite hopeful that this edition will be informative and reader-friendly. I am thankful to Jaypee Brothers Medical Publishers (P) Ltd., New Delhi, India for publishing this book.

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Acknowledgments

I would like to thank Mr Jitendar P Vij (Group Chairman), Mr Ankit Vij (Group President), Ms Chetna Malhotra Vohra (Associate Director), Ms Saima Rashid (Project Manager), Mr Umar Rashid (Development Editor) and Production team of M/s Jaypee Brothers Medical Publishers (P) Ltd., New Delhi, India.

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CHAPTER

1

Molecular Markers of Thyroid Cancer

Nelson George, Sabaretnam M, Amit Agarwal

INTRODUCTION

Thyroid cancer is the one among the top twenty cancers in the world and its occurrence rate is increasing every year. In US, as per the statistical report from the surveillance, epidemiology, and end result program, thyroid cancer is coming 8th position in data sheet of occurrence. However, the survival rates are higher while comparing to other cancers. In other parts of world also, there is a gradual increase in the thyroid cancer occurrence (Italy, India, UK, Australia, Korea, Japan, etc.). Meanwhile certain countries like Norway and Sweden, its prevalence is decreased. Based on recent data, thyroid cancer is the fifth most common cancer in women. There are four major types of thyroid cancers: (1) papillary thyroid cancer (PTC), (2) follicular thyroid cancer (FTC), (3) medullary thyroid cancer (MTC) and (4) anaplastic thyroid cancer (ATC). These types are classified based on their histopathological morphology and confirmed by the molecular markers. Although fine-needle aspiration biopsy (FNAB) and scanning are considered as important techniques for the diagnosis of thyroid nodules, there is still a large “gray zone” in these techniques. Therefore, it is clinically important to have molecular markers that in conjunction with FNAB can identify the subset of patients with subtypes and aggressive nature of tumor. Molecular markers are essential part in the confirmation of any cancers. Specific markers determine the type and aggressiveness of the cancer so that treatment will be targeted and efficient. The use of molecular markers for thyroid cancer diagnosis, prognosis, targeted therapy and surveillance has been an exciting area of study and change. Recent investigative focus on promising new markers will very likely lead to improvements in the diagnostic utility of FNAB in predicting malignancy, as well as provide more accurate prognostic information preoperatively and postoperatively.

MOLECULAR BIOLOGY OF THYROID CANCER

Many studies show the difference in gene expressions in relation to different mutation in PTC. Downregulation of TSHR, AIT (SLC5A8), TPO and Tg in PTCs with BRAF mutations. Sodium-iodide symporter (NIS) expressions

are very crucial in thyroid cancer. Impaired NIS trafficking to the cell membrane: expression of NIS was limited to cytoplasm and less on cell membrane (it is important that not only the expression level but also the localization of the transporter is crucial for its function). NIS expression levels are five times lower in BRAF positive tumors than BRAF negative tumors. SLC5A8 is down-regulated forty times through gene methylation in classic PTC.

SPECIFIC MOLECULAR MECHANISM INCLUDING THE SILENCING OF THYROID-SPECIFIC GENES

MAP Kinase Signaling Pathway

Mitogen-activated protein (MAP) kinase pathway plays a fundamental role in cell proliferation, differentiation, and apoptosis and survival. Mutations to the pathway cause continuous activation of pathway and leads to cancer. Three major mutations are involved.

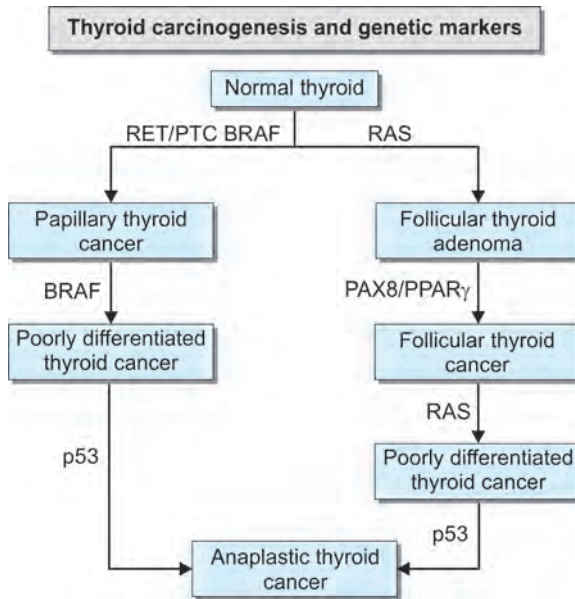
1. BRAF mutations
2. RAS mutations
3. RET/PTC rearrangements.

BRAF T1799A represents one of the most exciting advances in molecular biology of thyroid cancer. It is the most common known genetic alteration in thyroid cancer. This mutation causes a V600E amino acid change in protein, resulting in constitutive oncogenic activation through BRAF phosphorylation. Numerous studies have demonstrated the association of BRAF mutation with aggressiveness, tumor invasion, metastasis and recurrence of PTC as well as loss of radioiodine uptake has a close association with dedifferentiation of PTC and silencing of thyroid-specific genes. Therefore, BRAF mutation is a novel powerful prognostic marker for poorer prognosis of thyroid cancer and is equally helpful in finding the initial surgical and medical cause of thyroid cancer. Also it prevent an opportunity to reverse the process of BRAF mutant promoted silencing of thyroid-specific genes by silencing the expression of BRAF mutant in thyroid cells.

PI3K Signaling Pathway

PI3K pathway also plays a fundamental role in regulation of cell growth, proliferation and survival in thyroid cancer. This signaling can be antagonized by tumor-suppressor gene *PTEN* product, *PTEN* protein. Mutation to *PIK3CA* gene causes inactivation of *PTEN* gene, resulting in aberrant signaling of PI3/AKT pathway. *PIK3CA* mutation is common in FTC and ATC.

Genetic alterations that could activate both MAP kinase and PI3K pathways were found in 80% ATC, thus portraying toward extensive role of dual involvement of MAP kinase and PI3K pathways in pathogenesis of ATC. This supports a recent hypothesis that targeting multiple signaling pathways may be an effective and necessary therapeutic strategy for thyroid cancer.

Flowchart 1.1: Genetic markers of thyroid carcinomas.

Aberrant Gene Methylation in Thyroid Cancer

Changes in gene methylation leads to inappropriate silencing of genes particularly tumor-suppressor genes like *TIMP3*, *DAPK*, *SLC5A8*, *RARβ2*, *NIS*, *TSHR*, *SLC26A4*, *Tg* and *PTEN*. Aberrant signaling of both PI3 kinase and MAP kinase pathway provides a molecular basis for therapeutic strategies using demethylation agents for thyroid carcinoma. Also, the DNA methylation markers can be easily detected in serum and fine-needle aspiration cytology (FNAC) sample held great potential as novel diagnostic and prognostic molecular marker for thyroid cancer.

There are three major types of molecular markers: (1) genetic markers, (2) immunocytochemical markers and (3) epigenetic markers.

Genetic Markers

Genetic mutations are used in thyroid cancer as biomarkers. Many mutations are found in thyroid cancer, although few mutations are considered as markers of thyroid cancer. RAS sporadic mutations are the first identified markers (1987) for thyroid cancers. In 1990 sporadic RET/PTC translocations were first reported in PTCs, and in 1993, ATCs with *p53* mutation and NTRK1 mutations in PTC were first reported. PAX8/PPAR γ translocations were found in FTCs in 2000, followed by the discovery of BRAF mutations in 2003 for the first time (Flowchart 1.1).

BRAF mutation: There are three isoforms for RAF proteins in MAP kinases pathway; BRAF is the most active form of RAF protein. The mutation occurs at exon 11 and 15. In fact, BRAF V600E is considered as the most occurring mutation (90% of BRAF mutations). BRAF mutations are the common mutations in thyroid cancer, especially in the PTC which accounts for the 80% of total thyroid cancers. BRAF mutations are present in the PTC 30–90% in different areas and populations. It is also present in other thyroid cancers. This indicates capability of BRAF as a biomarker of thyroid cancers particularly PTC which accounts 90% of thyroid cancers. Sixteen studies from 2003 to 2007 showed the positive correlation of BRAF with PTC and these studies suggested BRAF as a potent marker of PTC. After that, many researchers and diagnostics started using BRAF as a marker of PTC. All of these studies are from Europe, Russia, Asia and USA, meanwhile there was only few studies from India (recently, Mosin S Khan et al. 2014 showed 25% occurrence of BRAF mutations in Kashmiri population). Our data (unpublished) shows 70% BRAF V600E mutations in an endemic goiter area of North India. Most of the data suggest the presence of BRAF mutations in different stages and types of PTC, although there are controversies since few studies accounts the presence of BRAF in PTCs irrespective of their aggressiveness and progression of cancer. Recent studies of BRAF positivity on FNAC samples reveals 99.8% chances of thyroid cancer malignancy.

RAS mutations: RAS gene have three isoforms: HRAS, NRAS and KRAS. RAS mutations are commonly present in the FTCs rather than PTC which have high frequency of BRAF mutations. RAS mutations have been suggested to be a biomarker for a more aggressive form of thyroid cancer. In fact, follicular variant of PTC has high incidence of RAS mutations (especially NRAS mutations). Zhaowen Zhu et al. (2003) have shown 43% occurrence of RAS mutations in follicular variant of PTCs. There are many RAS mutation detection kits available with diagnostic features (ABI, Roche, etc.). This indicates importance of RAS mutations as a molecular marker in FTCs. Although RAS mutations are present in thyroid cancers, it cannot be reliably used to differentiate between benign and malignant tumors. But, RAS mutations help a clinician in predicting a chance of malignancy as tumor progresses.

RET/PTC rearrangements: RET/PTC (rearranged in transformation/papillary thyroid carcinoma) is formed as a fusion kinase from chromosomal translocation analysis. The protein acts as a thyroid-specific oncogenic kinase, and it develops spontaneous PTCs. RET/PTC rearrangements are the cause of sporadic thyroid cancers and RET gene is fused with 15 other genes; this causes continuous activation of MAP kinase pathway and further it leads to thyroid cancer. RET/PTC1 and RET/PTC3 are the commonly occurring rearrangements arising from chromosome 10 inversions.

PAX8-PPAR γ gene fusion: The peroxisome proliferator-activated receptors (PPARs), including α , β , δ , and γ subtypes, are part of the ubiquitous nuclear

hormone receptor super family. Discovery of the PAX8/PPAR γ translocation in follicular thyroid carcinoma has promoted progress in the role of PPAR γ as a tumor-suppressor and potential therapeutic target. Rearrangements involving PAX-8 and PPAR γ were reported as specific for FTC (63% of the cases). More recently, PAX8/PPAR γ was detected by RT-PCR in 25–56% of FTC and in 0–13% of follicular adenomas. Altered PPAR γ activity has subsequently been shown to have a potential role in several types of thyroid cancer.

p53 tumor-suppressor: *p53* is an important tumor suppressor that regulates cell cycle arrest and apoptosis. *p53* has major role in halting cell cycle when a cell is damaged, and it allows to get repaired or to initiate apoptosis to prevent damaged cells from converting into tumors. Mutations that cause inactivation of the *p53* gene may be found in up to 50% of all human malignancies making it the most common mutation associated with human cancer. *p53* are involved in the initial events of thyroid cancers and makes the thyroid cell in to undifferentiated tumors. This mechanism is present in case of anaplastic and poorly differentiated thyroid cancers. It indicates the aggressiveness of tumor. But, its sensitivity is too low to be used as a regular marker.

Immunocytochemical Markers

Thyroid cancer is the most common endocrine malignancy and more than 95% of thyroid carcinomas originate from follicular epithelial cells. The incidence of thyroid carcinomas derived from follicular cells varies worldwide depending on dietary iodine intake, but in most countries, it has increased during the past few decades, and in North America, it is one of the most rapidly increasing cancers, representing a major cause of morbidity in premenopausal women. Medullary carcinomas that originate from parafollicular C cells, which are involved in the production of calcitonin are rare, representing only about 3% of thyroid tumors. Most follicular cell-derived carcinomas are well-differentiated malignancies that can be effectively treated by surgical resection with or without radioactive iodine (RAI) ablation (Flowchart 1.2).

Galectins: Thyroid cancer is one of the few cancer types that remain a diagnostic dilemma for the clinician as thyroid nodules are extremely common in the general population which are identified in 5% of patients by palpation and 50% by ultrasound examination. FNAB is the most trusted initial diagnostic test for evaluation of thyroid nodules. Still, diagnosis of thyroid cancer remains uncertain in a large number of cases.

Recently, galectin-3 (Gal-3) is found as the most accurate marker for differentiated thyroid cancer diagnosis (DTC) when compared with a panel of 56 other molecular markers. Galectins are a large family of proteins that recognize and bind galactosides on cell glycoproteins and glycolipids. Gal-3 is a structurally unique 31-kDa member of the galectin family which is localized in the nucleus, cytoplasm and extracellular space (Fig. 1.1). Many studies

Flowchart 1.2: Immunocytochemical markers of thyroid carcinomas.

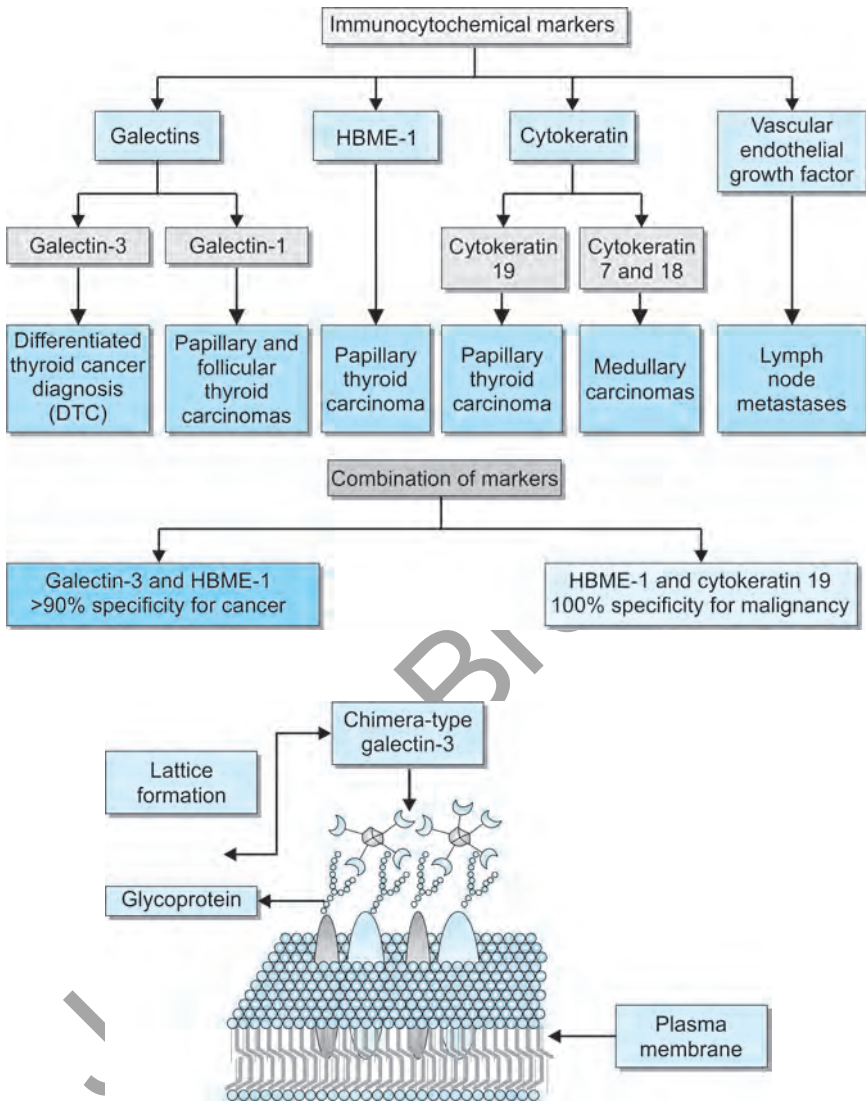


Fig. 1.1: Mode of interaction of galectins.

have shown Gal-3 as a potent mediator of transforming cells into malignant phenotype. It has been found that Gal-3 works as an up regulator of thyroid-specific transcription factor-1, which is localized in nucleus, thereby contributing to highly proliferative state of proliferating human thyroid papillary carcinoma cells. Along with Gal-3 studies have shown overexpression of galectin-1 in all papillary and follicular thyroid carcinomas, which were derived from follicular epithelium, as compared to normal thyroid tissue and benign thyroid adenomas.

Hector Battifora mesothelial cell: Hector Battifora mesothelial cell (HBME-1) is a marker of mesothelial cells. Several studies have demonstrated its utility as a marker of malignant thyroid tumors of follicular epithelial derivation. Nechifor-Boila et al. showed more than 95% HBME-1 expression in PTC samples whereas in normal thyroid tissues, there is virtually no expression of HBME-1, making it a potent marker for diagnosis. Expression of HBME-1 was also noted in benign thyroid lesions such as nodular goiter and lymphocytic thyroiditis, usually in a focal staining fashion with a reported overall positive rate of 26%, 12% and 19%, respectively. Reports have shown HBME-1 as consistently reliable markers in the distinction of papillary from nonpapillary neoplasms and non-neoplastic lesions when used in combination with CK19.

Cytokeratin-19: Depending on molecular weight of different keratin filaments, various types of CKs have been found. CK1, CK4, CK10 and CK13 are detected in stratified squamous epithelium whereas CK7, CK8, CK18 and CK19 are found in simple or glandular epithelium. Different antibodies directed against CKs have been used as a useful method to identify differential expression patterns in normal parenchyma, benign nodules and malignant tumors. Reports have shown expression of CK19 in papillary carcinomas with strong diffuse cytoplasmic reactivity in 80–100% cases. CK19 has been found more specific in detection of PTC than other follicular adenomas and follicular carcinomas with its distribution and intensity of expression being major players in accurate detection. The reported sensitivity and specificity using CK19 as a single marker is as high as 92% and 97%, respectively. The use of CK19 immunolocalization in cell-block preparation of thyroid aspirates has also been reported to aid in accurate diagnosis of malignancy in cytomorphologically equivocal cases of PTC.

Other than in PTC, reports have shown medullary carcinomas showing strong positive staining for CK7 and CK18 in 77% of cases and only focal staining for CK19 in 69% of lesions. The use of CK19 immunolocalization in cell-block preparation of thyroid aspirates has also been reported to aid in accurate diagnosis of malignancy in cytomorphologically equivocal cases of PTC.

Vascular endothelial growth factor: Vascular endothelial growth factor (VEGF) being the most potent inducer of neovasculature, appears to be related to different aspects of thyroid tumor behavior. The increased expression of VEGF have been correlated with thyroid neoplasia with a higher risk of recurrence and metastasis using immunohistochemical staining of VEGF proteins and Northern blot analysis of VEGF messenger RNA (mRNA). The elevated expression of VEGF mRNA in thyroid cancers have been associated with high tumorigenic potential, which could be an important event in the transition from low- to high-grade tumors. Lymph node metastases of thyroid tumors showed increased VEGF expression with respect to the primary

tumor. The report has shown higher VEGF expression in metastatic cancer compared with nonmetastatic cancers on quantitation of VEGF expression by the percentage of stained thyrocytes and the intensity of immunostaining in PTC.

Epigenetic Markers

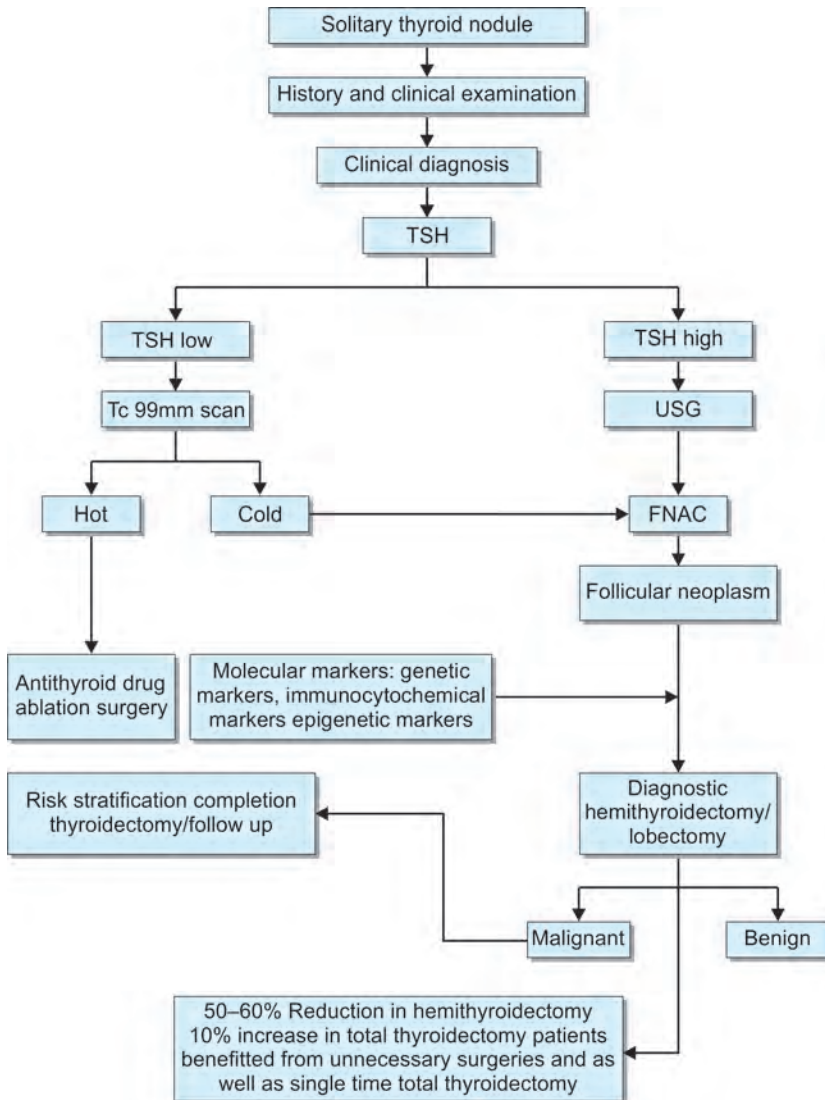
Epigenetics is defined as the study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence. Mechanisms involved in of epigenetic regulations are DNA methylation, histone protein modification, nucleosome positioning and microRNA silencing. There are studies on DNA methylation, histone modifications and microRNA silencing in thyroid cancer. Epigenetic defects can be easily reversed by using therapeutic agents. Recent advancements in the epigenetic modifications are microRNAs. They are single-stranded 20–25 bp long nucleotides. Single miRNA can regulate multiple genes and the first study of miRNA in thyroid cancer was in 2005 (17 miRNAs were overexpressed and 5 miRNAs were under expressed). Studies showed upregulation of miRNAs 146b, 221, 222, 224, 155 and 181b in PTC, and few studies showed the upregulation miRNAs 155, 187, 221, 222 and 224 in FTCs.

APPLICATIONS OF MOLECULAR MARKERS

Diagnosis

Molecular markers play a key role in taking decisions in thyroid surgeries. The efficiency and accuracy of diagnosis improves the surgery. So far, the studies show a reduction in the hemithyroidectomies and are helpful since the diagnosis through molecular markers is advanced. There are studies saying BRAF mutational detection in PTCs helps the surgeon in choosing the particular nodules, which are affected and can be removed specifically. Galectin immunocytostaining in FNAC sample of thyroid is regularly used; studies help in discriminating benign from malignant thyroid lesions. Along with genetic markers, somatic mutation testing, *mRNA* gene expression platforms, protein immunocytochemistry and miRNA panels have improved the diagnostic accuracy of indeterminate thyroid nodules; although no test is perfectly accurate.

Fine-needle aspiration biopsy cannot distinguish between benign and malignant nonpapillary follicular neoplasms. Both benign and malignant lesions appear similar in cytologic specimens. The diagnostic terminology such as “follicular neoplasm” reflects the limitations of thyroid cytology, since the diagnosis of follicular carcinoma is only based on the demonstration of capsular and/or vascular invasion. Several authors have shown that, at most, only 20–30% of cases diagnosed as “follicular neoplasm” are diagnosed as follicular carcinoma on histological examination and a majority is composed of follicular adenomas and cellular adenomatoid nodules (Flowchart 1.3).

Flowchart 1.3: Efficacy of molecular marker in the management of indeterminate nodules.

Targeted Therapy

In advanced papillary thyroid carcinoma and poorly differentiated and anaplastic carcinoma, the outcome is dismal because the thyroid cells lose the ability to take up RAI and thus cannot be destroyed. Recent understanding of tumor biology has shown that this inability to concentrate iodine is due to the silencing of specific thyroid genes like the *NIS*, *AIT*, *TSHR*, *PENDRIN* and *TPO*, which are involved in the iodine uptake and its organification. Silencing of these genes results from aberrant activation of the MAP kinase

Table 1.1: Targeted therapy and therapeutic agents.

Therapeutic agent	Mechanism of action	Outcome	Common adverse effects
Doxorubicin	Cytotoxic chemotherapy	37% PR	Cardiomyopathy, acute arrhythmias, granulocytopenia, nausea, infertility, alopecia
Sunitinib	Non-specific TKI	14% PR	Neutropenia, diarrhea, hand/foot syndrome and leucopenia
Sorafenib	Non-specific TKI	12.2% PR	Hand/foot syndrome, diarrhea, alopecia, fatigue, weight loss, rash, anorexia, nausea 74% SD
Vemurafenib	BRAF V600E small molecule inhibitor	3 patients: • 1 patient with PR • 2 patients with SD	Cutaneous squamous cell carcinomas and keratoacanthomas, rash, fatigue, arthralgias, nausea
Selumetinib	Elective MEK inhibitor to induce iodine re-uptake	40% (8/20) treated with RAI: • 5 patients with PR • 3 patients with SD	Fatigue, rash, liver function test elevations

(PR: Partial response; RAI: Radioactive iodine; SD: Stable disease; TKI: Tyrosine kinase inhibitor).

and/or PI3/Akt pathway, which results in tumorigenesis and dedifferentiation of thyroid cancer. Aberrant activation of these crucial genetic pathways results from mutation in BRAF, N-K RAS and *RET* oncogenes. Thus, with improved understanding of the genetic alterations involved in thyroid carcinogenesis, new options for advanced thyroid cancer patients have emerged including tyrosine kinase and small-molecule inhibitors, and targeted inhibition of upregulated pathways to induce iodine reuptake. Tyrosine kinases have a pivotal role in tumor proliferation, angiogenesis and metastasis. Tyrosine kinase inhibitors non-specifically target these pro-oncogenic kinases including VEGFR-1, VEGFR-2, EGFR, PDGFR, MET, FGFR, in addition to RAF and RET (Table 1.1).

Demonstration of disease progression represents the main indication for referring iodine-refractory DTC patients for treatment with kinase inhibitors (KIs). The most studied KI for treatment of RAI refractory DTC is sorafenib. The efficacy results of the first phase-III trial of any KI in DTC—the DECISION study led to the FDA approval of sorafenib for treatment of progressive RAI refractory DTC. The phase III trial of another molecule *lenvatinib*, which targeted RET, AKIt, VEGFRs, FGFRI and PDG FRbeta, known as the SELECT trial led to its global approval for use in DTC. Another promising molecule is *selumetinib*, which is currently in phase III trials. *Vandetanib*, which targets RET, VEGFR-2, VEGFR-3 and EC1FR, has been approved by FDA for use in advanced MTC.

SURVEILLANCE

Serum thyroglobulin (Tg) levels remains the only blood test available for assessment of disease status in thyroid cancer patients undergoing long-term follow-up and is unreliable for interpretation in 20–30% of patients because of Tg antibodies. Detection of TSHR mRNA in peripheral blood has potential use as an additional postoperative indicator of persistent or recurrent thyroid cancer. Another useful marker of recurrence of thyroid cancer is measurement of peripheral BRAF mutation.

PROGNOSIS OF THYROID CANCERS

In fact, the most common types of thyroid cancer (PTC and FTC) are the most curable. In younger patients, both papillary and follicular cancers have a more than 97% cure rate if treated appropriately. Both papillary and FTCs are typically treated with complete removal of the lobe of the thyroid that harbors the cancer in addition to the removal of most or all of the other side.

Apart from the normal BRAF, RAS, RET/PTC mutations markers of aggressive thyroid cancers also include mutations in p53 (25–30%), PIK3CA (10–20%), CTNNB1 (10–20%) and AKT1 (5–10%). Telomerase reverse transcriptase (TERT) promoter mutations are new advancement in the molecular marker of aggressive thyroid cancers. In thyroid cancers, TERT promoter mutations were identified in 7–22% of PTC and 35% of FTC, were often found in association with BRAF or RAS mutations and were more likely in patients with histologically aggressive DTC.

Research over biomarkers is extensively done, and it helps in improving the survival rates of patients. It helps in the diagnosis and tracing the progression of tumor. Setting a marker in any of the malignancy requires several criteria including uniqueness in occurrence, prevalence of marker, sensitivity, etc. Once all these criteria match then the marker is used in a clinical setup. Also a team of biologists, geneticists, statisticians, oncologists, surgeons and even engineers are required for finding new biomarkers. Combination of biomarkers is also important to elucidate the chances of false predictions in diagnosis. New areas of research like metabolomics and epigenetic changes keep the search awake for the new molecular markers.

In conclusion, thyroid cancer is the one among the top twenty cancers in the world and its occurrence rate is increasing every year. Molecular markers are of mainly three types: genetic, immunocytochemical and epigenetic. Common genetic markers are BRAF, RAS, P53 mutations and RET/PTC, PAX8-PPAR γ translocations. Immunocytochemical markers used in diagnosis are galectins, HBME-1, cytokeratin-19 and VEGF. Use of microRNA as a molecular marker is new advancement in diagnosis. About 10–25% of FNABs give an indeterminate result as follicular lesion, follicular lesion of undetermined significance. Although many markers are developed, none of them is 100% efficient but can be used for “rule-in and rule-out” tests that attempt to

exclude or confirm. Rule-in tests are like point mutations (BRAF and RAS) or gene arrangements (RET/PTC, PAX8-PPAR γ). It may help to predict cancer. Molecular marker testing is in its advanced state of new technologies, and it has to be developed by a team of clinicians and biologists.

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