Chapter 2

Molecular Alterations and Expression Dynamics in the Etiopathogenesis of Thyroid Cancer

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Abstract

Thyroid carcinoma is the most prevalent endocrine malignancy and accounts for 2% of all human cancers. In the past decade, knowledge of genetic alterations of thyroid cancer (TC) has rapidly expanded, which has provided new insights into thyroid cancer etiology and has offered novel diagnostic tools and prognostic markers that enable improved and personalized management of thyroid cancer patients. Alterations in key signaling effectors seem to be the hallmark of distinct forms of thyroid neoplasia. Mutations or rearrangements in genes that encode Mitogen activated protein kinase (MAPK) pathway effectors seem to be required for transformation. Mutations in *BRAF* were the most recently identified MAPK effector in thyroid cancer. *BRAF* V600E is the most common alteration in sporadic papillary carcinoma. Three *RAS* proto-oncogenes (*NRAS, HRAS* & *KRAS*) are implicated in human thyroid tumorigenesis. High incidence of thyroid cancer worldwide indicates the importance of studying genetic alterations that lead to its carcinogenesis. *BRAF* and *RAS* alterations represent a novel indicator of the progression and aggressiveness of thyroid carcinogenesis. The GSα-adenylyl cyclase-cyclic AMP (cAMP) cascade is effected in thyroid cancer. Promoter hypermethylation of multiple genes especially *TSHR* has been identified to play a role in thyroid cancers, in particular showing a close association with *BRAF* mutational status. So, the main aim of the study was to elucidate the involvement of *BRAF* and *RAS* gene mutations along with BRAF expression and thyroid-stimulating hormone receptor (TSHR) hypermethylation in North Indian patients and investigate their association with clinicopathological characteristics.

We screened exon 15 of *BRAF* gene and exons 1 and 2 of *RAS* genes (*HRAS, KRAS*, and *NRAS*) in 60 consecutive thyroid tissue (tumor and adjacent normal) samples. Overall mutations in *BRAF* were found to be 25% (15 of 60) affecting codon 600 (valine to glutamine) and restricted only to papillary thyroid cancer and well-differentiated grade.

BRAF mutations were significantly associated with well-differentiated disease and elevated thyroid-stimulating hormone (TSH) levels ($P < 0.05$). Overall, increased expression of BRAF was found in 90% (54 of 60) of thyroid cancer cases and significantly associated with nonsmokers. Totally, 86.7% (13 of 15) of *BRAF* mutation-positive patients were having BRAF protein overexpression compared to 91.2% (41 of 45) of patients with wild-type *BRAF* status ($P > 0.05$). We screened 60 consecutive thyroid tumor and adjacent normal tissues for mutations, if any, in the exons 1 and 2 of *RAS* genes (*HRAS, KRAS*, and *NRAS*) and 140 blood samples from thyroid cancer patients for *HRAS* T81C polymorphism in codon 27 in comparison with 170 cancer-free controls from a Kashmiri population. Thyroid tumor tissue samples were devoid of any mutation, but a frequent nucleotide change at position 81 (T > C) in exon 1 of *HRAS* gene was seen. In *HRAS* T81C SNP, frequencies of TT, TC, and CC genotypes among cases were 41.4, 38.6, and 20.0%, while in controls genotype frequencies were 84.1, 11.7, and 4.2%, respectively. A significant difference was observed in variant allele frequencies (TC + CC) between the cases and controls (58.6 vs. 16%) with odds ratio of 7.4 (CI = 04.3–12.7; $P < 0.05$). Interestingly, combined TC and CC genotype abundantly presented in follicular thyroid tumor ($P < 0.05$). Moreover, a significant association of the variant allele (TC + CC) was found with nonsmokers ($P < 0.05$). *TSHR* gene was found to be hypermethylated in 25% (15 of 60) of the cases with strong association with elevated TSH levels (OR = 4.0, P = 0.02). *TSHR* promoter was hypermethylated in 73.3% (11 of 15) of patients with *BRAF* V600E mutation compared to 26.7% (4 of 15) of patients having absence of *TSHR* promoter methylation and the association was significant ($P < 0.05$).

We conclude that both mutational events and overexpression of *BRAF* gene are highly implicated in pathogenesis of thyroid cancer and the BRAF protein overexpression is independent of the *BRAF* mutational status of thyroid cancer patients. *RAS* gene mutation does not prevail in this population. Contrary to this, *HRAS* T81C polymorphism moderately increases thyroid cancer risk with rare allele as a predictive marker for follicular tumors. Our study showed a high implication of *TSHR* gene methylation and its significant association with *BRAF* V600E mutation in thyroid tumors, depicting a positive connection between *TSHR* pathway and MAP kinase pathway.

Keywords: polymerase chain reaction, papillary thyroid cancer, thyroid-stimulating hormone, benign thyroid disease, lymph node metastasis, follicular thyroid cancer, mutation, polymorphism, gene, hypermethylation, genotype, expression

1. Introduction

Thyroid gland is the largest endocrine gland comprised of follicular cells and C cells. It synthesizes, stores, and secretes triiodothyronine (T3) and thyroxine (T4) (**Figure 1**). Follicular cells comprise most of the epithelium and are responsible for iodine uptake and thyroid hormone synthesis. C cells are dedicated to the production of the calcium-regulating hormone calcitonin [1].

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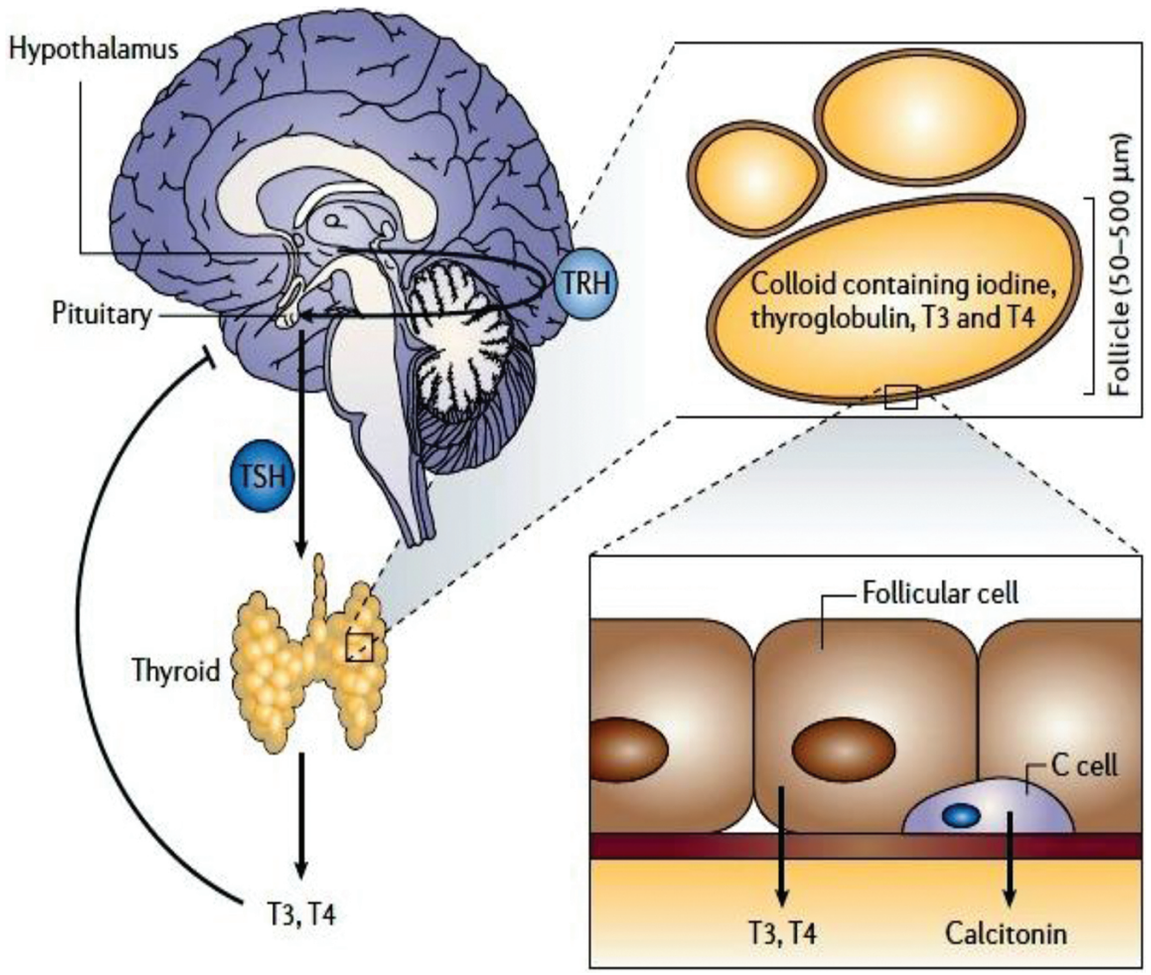

Figure 1. The thyroid gland.

At molecular level, cancer is caused by molecular defects in cell function resulting from common types of alterations to a cell's genes. Cancer is a disease of abnormal gene expression which may occur due to DNA mutation, translocation, amplification, deletion, loss of heterozygosity, etc. The overall result is an imbalance of cell replication and cell death that leads to unregulated growth and spread of cells in different parts of body [2, 3].

Thyroid cancer (TC) typically occurs in *thyroid nodules* and is relatively common, occurring in 6% of adult women and 2% of adult men which can be detected by palpation and imaging in a large proportion of adults. Approximately 90% of thyroid malignancies are well-differentiated thyroid carcinomas arising from thyroid follicular epithelial cells, which are classified as papillary or follicular based on histopathological criteria, whereas 3–5% of cancers originate from parafollicular or C cells. Follicular adenoma is a benign tumor that may serve as a precursor for some follicular carcinomas. Recurrence occurs in 20–40% of patients in spite of the fact that differentiated thyroid carcinomas are usually curable by the combination of surgery, radioiodine ablation, and thyroid-stimulating hormone suppressive therapy [4] due to cellular dedifferentiation which is accompanied by more aggressive growth, metastatic

spread, and loss of iodide uptake ability, making the tumor resistant to the traditional therapeutic modalities and radioiodine [5]. Knowledge of genetic alterations occurring in thyroid cancer has rapidly expanded in the past decade. This improved knowledge has provided new insights into thyroid cancer etiology and has offered novel diagnostic tools and prognostic markers that enable improved and personalized management of patients with thyroid nodules [6].

TC is the most common malignancy of the endocrine system. It accounts for approximately 2% of all newly diagnosed cancer cases and majority of endocrine cancer related deaths each year [7, 8]. An estimated 12.66 million people were diagnosed with cancer across the world in 2008, and 7.56 million people died from the disease. This equates to around 188 cases for every 100,000 people (using the crude rate). Among the 20 most commonly diagnosed cancers worldwide, thyroid cancer figures on 17th (2% of all cancers) number (2008 estimates) [9]. There were 213,179 new thyroid cancer cases and 163,000 cases among females worldwide by the year 2008 [10]. Its prevalence continues to rise; in 2008, it became the sixth most diagnosed cancer among women in United States Around 56,460 cases (men – 13,250, women – 43,210) and 1780 deaths (men – 780, women – 1000) from thyroid cancer occurred in 2012 [11]. The data indicated that there were 60,220 new cases in 2013, accounting for 3.6% of all new cancer cases. There were 1850 thyroid cancer-related deaths in 2013, accounting for 0.3% of all cancer deaths. There are currently ~534,973 TC patients in USA. The reasons for increased incidence are unclear, with potential explanations including increased screening, more widespread diagnostic testing of asymptomatic thyroid nodules, changing demographics, and environmental risk factor. TC accounts for approximately 10% of malignancies diagnosed in persons aged 15–29 years. Follicular cancers include papillary thyroid cancer (PTC, 80%), follicular thyroid cancer (FTC, up to 11%), Hürthle cell cancer (3%), and anaplastic thyroid cancer (ATC, 2%). Medullary thyroid cancer (MTC) accounts for about 4% of thyroid cancers [12]. As expected from the size of Asia's population, the majority of cancer cases occurred there. Between 1984 and 1993, over 5614 thyroid cancer cases were recorded in India which included 2007 males and 3617 females and the age standardized rate (ASR) in 1993 was 1.0/year/10^5 and 1.9/year/10^5 for males and females, respectively [13]. The age-adjusted incidence rates of thyroid cancer per 100,000 are about 1 for males and 1.8 for females as per the Mumbai Cancer Registry, which covered a population of 9.81 million subjects. The commonest cancer type was papillary, followed by follicular cancer. TC is the 8th most common cancer in the valley of Kashmir and 7th most common cancer among women of Kashmir valley. Among all types of cancers in the Kashmir valley, the frequency of TC has increased from 2.3% in 1995 to 5.4% in 2010, keeping overall frequency of 3.2% [14].

2. Classification of thyroid tumors

The classification of thyroid tumors is given by the World Health Organization (WHO) and Armed Forces Institute of Pathology (AFIP) with slight difference [15]. According to AFIP, priority is given to the cell of origin and incorporating, in each cell type, special tumor types

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and subtypes designated as "variants". Classification scheme adopted by the Armed Forces Institute of Pathology (AFIP) is depicted in **Figure 2**.

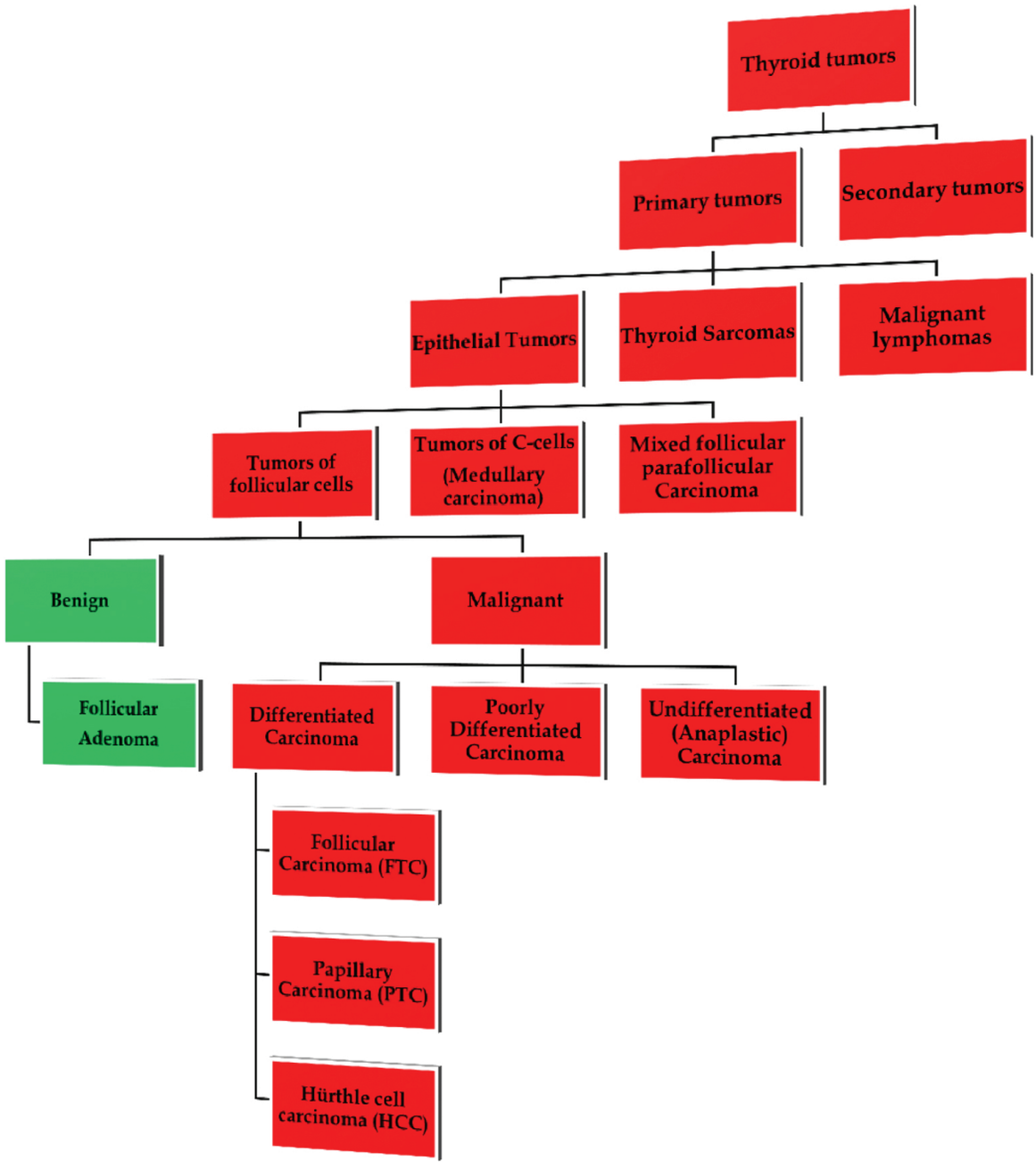

Figure 2. Classification of thyroid tumors as per Armed Forces Institute of Pathology (AFIP).

3. Staging of thyroid carcinoma

There are different stages of TC as designated by the American Joint Committee on Cancer (AJCC) [15]. The SEER modified 7th edition AJCC staging is given in **Tables 1** and **2**.

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor is found
T1	Tumor size ≤ 2 cm in greatest dimension and is limited to the thyroid
T1a	Tumor ≤ 1 cm, limited to the thyroid
T1b	Tumor > 1 cm but ≤ 2 cm in greatest dimension, limited to the thyroid
T2	Tumor size > 2 cm but ≤ 4 cm, limited to the thyroid
T3	Tumor size > 4 cm, limited to the thyroid or any tumor with minimal extrathyroidal extension (e.g., extension to sternothyroid muscle or perithyroid soft tissues)
T4a	Moderately advanced disease; tumor of any size extending beyond the thyroid capsule to invade subcutaneous soft tissues, larynx, trachea, esophagus, or recurrent laryngeal nerve
T4b	Very advanced disease; tumor invades prevertebral fascia or encases carotid artery or mediastinal vessel
All anaplastic carcinomas are considered stage IV:	
T4a	Intrathyroidal anaplastic carcinoma
T4b	Anaplastic carcinoma with gross extrathyroid extension
Regional lymph nodes (N)	
Regional lymph nodes are the central compartment, lateral cervical, and upper mediastinal lymph nodes:	
NX	Regional nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
N1a	Metastases to level VI (pretracheal, paratracheal, and prelaryngeal/Delphian lymph nodes)
N1b	Metastases to unilateral, bilateral, or contralateral cervical (levels I, II, III, IV, or V) or retropharyngeal or superior mediastinal lymph nodes (level VII)
Distant metastasis (M)	
M0	No distant metastasis is found
M1	Distant metastasis is present

Table 1. TNM classification for thyroid cancer (SEER modified 7th edition AJCC staging).

Stage grouping			
Separate stage groupings are recommended for papillary or follicular (differentiated), medullary, and anaplastic (undifferentiated) carcinoma			
Papillary and follicular thyroid cancer (age < 45y):			
Stage	**T**	**N**	**M**
I	Any T	Any N	M0
II	Any T	Any N	M1
Papillary and follicular; differentiated (age ≥ 45y):			
Stage	**T**	**N**	**M**
I	T1	N0	M0
II	T2	N0	M0

Stage grouping			
Separate stage groupings are recommended for papillary or follicular (differentiated), medullary, and anaplastic (undifferentiated) carcinoma			
III	T3	N0	M0
IVA	T1-3	N1a	M0
	T4a	N1b	M0
IVB	T4b	Any N	M0
IVC	Any T	Any N	M1
Anaplastic carcinoma (all anaplastic carcinomas are considered stage IV):			
Stage	**T**	**N**	**M**
IVA	T4a	Any N	M0
IVB	T4b	Any N	M0
IVC	Any T	Any N	M1
Medullary carcinoma (all age groups):			
Stage	**T**	**N**	**M**
I	T1	N0	M0
II	T2, T3	N0	M0
III	T1–T3	N1a	M0
IVA	T4a	N0	M0
	T4a	N1a	M0
	T1	N1b	M0
	T2	N1b	M0
	T3	N1b	M0
	T4a	N1b	M0
	T4a	N0, N1b	M0
	T1-T4a	N1b	M0
IVB	T4b	Any N	M0
IVC	Any T	Any N	M1

Table 2. Stage grouping of thyroid cancer (SEER modified 7th edition AJCC staging).

4. Risk factors of thyroid cancer

4.1. Gender and age

Females exhibit a better prognosis than men. TC is 2–4 times more frequent in women. It is rare in patients aged <16 years, presenting an annual incidence of 0.02–0.3/100.000 [16, 17]. Its incidence increases with ageing, and the average age at diagnosis is 45–50 years.

4.2. Ethnic differences

TC incidence has a geographic and ethnic variability. The incidence of TC in areas such as Iceland, Hawaii, the Philippines, Japan, and Israel is higher than in North America, Canada, and US. In US, the TC is more frequent in Caucasian descent subjects. All these findings suggest that such differences may be attributable to both environmental (e.g., dietary habits) and genetic factors [18].

4.3. Previous exposure to ionizing radiation

Previous exposure to ionizing radiation for external irradiation of the neck increases the incidence of thyroid nodules, either benign or malignant. Palpable nodules are detected in 20–30% of people exposed to radiation and in pediatric patients undergoing radiation therapy for oncological and hematological malignancies such as lymphoma or leukemia [19, 20].

4.4. Age at the time of irradiation

Irradiation is no longer an increased risk after 15–20 years of age. In children exposed to doses of 1 Gy, the excess risk for TC is equal to 7.7 [21]. Several studies have shown an increased risk of TC in children aged between 5 months and 10 years after the Chernobyl nuclear disaster [22].

4.5. Previous history of benign thyroid disease (BTD)

People with benign thyroid conditions like an enlarged thyroid (goiter), thyroid nodules (adenomas), and inflammation of the thyroid (thyroiditis) are more likely to develop thyroid cancer. Approximately 1 in 5 thyroid cancers (20%) occur in people who have had a BTD in the past [23].

4.6. Contribution of iodine in the food

In areas of sufficient iodine intake, PTC is more prevalent (80% of TCs), whereas in iodine-deficient areas, FTCs and ATCs are 2–3 times more frequently reported as compared to areas with adequate iodine intake [24].

4.7. Body mass index

High body mass index (BMI) has been shown as a risk factor for TC according to several case-control studies. There is a fivefold risk in obese men and 2 times in obese women. In postmenopausal women, weight gain of >14% positively correlates with the onset of TC [25].

4.8. Hormonal factors

According to the period of life in which thyroid cancer occurs, the female:male incidence ratio is different. In women of child bearing age, this ratio is about 4:1 and 1.5:1 in older, prepuberal,

and menopause individuals [26]. TSH regulates the growth and function of the thyroid gland [27]. Growth of some thyroid cancers is dependent on TSH secretion and suppression of TSH release by administration of thyroxin is often an effective treatment for thyroid carcinomas. The thyroxine-binding globulin level in normal females is 10–20% higher than in males and in pregnancy, a 50% increase in the level of thyroxine-binding globulin results in a similar magnitude increase in TSH level [28]. It therefore appears likely that TSH levels of non pregnant normal females will be elevated above the level in males at some point in the menstrual cycle although not necessarily throughout the cycle. An elevated risk was also reported in women who used estrogens for gynecological problems. In some studies, higher levels of estrogen receptors (ERs) were found in neoplastic than in normal thyroid tissues [29]. The ligand-bound dimer ER can interact with an estrogen-responsive element, resulting in transcriptional activation of the target gene [30]. 17 β-estradiol stimulates cell cycle progression early in G1 phase by induction of cyclin D1 gene expression. In different cell lines, the induction of cell growth was found to correlate with increased expression of cyclin D1 protein levels [31].

4.9. Smoking status

Although relatively little is known about the etiology of thyroid cancer beyond its association with radiation exposure and some previous thyroid disorders [32], data are slowly accumulating as to the protective effect of cigarette smoking on this disease. Thyroid cancer has been negatively associated with cigarette smoking in a number of studies, possibly consistent with the greater occurrence of the disease in women than in men [33]. There are at least five distinct proposed mechanisms for the effect of tobacco smoke on thyroid function. The *first* one relates to a smoking-related reduction in TSH secretion, as it has long been hypothesized that elevated levels of TSH may increase the risk of thyroid cancer. The lower body weight among smokers compared to nonsmokers is a *second* proposed explanation, as increased body weight was associated with a slightly increased thyroid cancer risk in the above-mentioned pooled analysis. A *third* possible biological pathway lies in the potential anti-estrogenic effect of cigarette smoke; a role for estrogen in the etiology of thyroid cancer is hypothesized because of the higher incidence of this cancer in females relative to males [34]. The *fourth* is higher levels of thyroxine-binding globulin and testosterone among smokers compared to nonsmokers and the *fifth* is the higher levels of thyrotoxins in tobacco smoke in heavy smokers compared to light and moderate smokers [35].

4.10. Oxidative stress

Oxidative stress (OS) is a state of excessive free radicals and reactive metabolites. In essence, OS represents an imbalance between the production of oxidants and their elimination by anti oxidative systems in the body. Many studies have linked OS to thyroid cancer by showing its association with abnormally regulated oxidative or antioxidative molecules [36].

5. Molecular biology of thyroid cancer

Thyroid tumors represent an appropriate model for the study of epithelial neoplastic transformation. The roles of somatic mutations, gene rearrangement (s), and level of gene expression in carcinogenesis are now well established. The application of molecular techniques to thyroid tumors has focused particular attention on the role of point mutations activating (or inhibiting) the genes for the TSH receptor (*TSHR*), *RAS, BRAF, Gsp, P53*, etc, specific rearrangements of the oncogenes *RET* and *TRK* and alterations in the pattern of expression of the oncogene *BRAF, MET*, etc [37]. The theory of sequential progression of well-differentiated thyroid carcinoma to poorly differentiated and undifferentiated thyroid carcinoma is because of genetic imbalances [38]. **Figure 3** depicts the model of multi-step carcinogenesis of thyroid neoplasms.

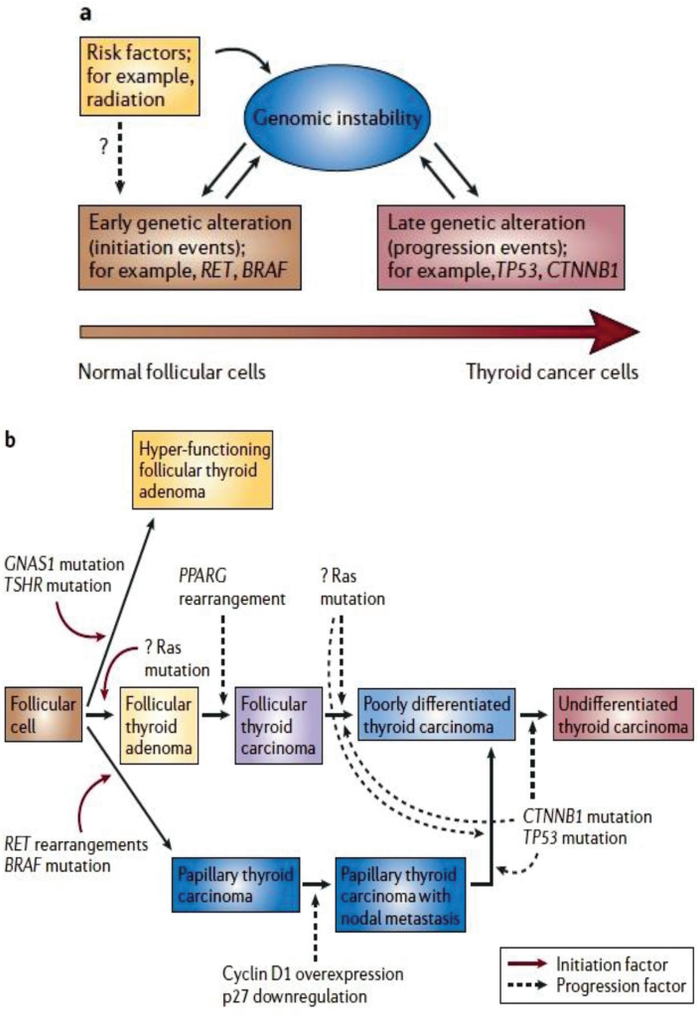

Figure 3. Model of multi-step carcinogenesis of thyroid neoplasms. The proposed model of thyroid carcinogenesis is based on general concepts and specific pathways. (a) Risk factors, such as exposure to radiation, induce genomic instability through direct and indirect mechanisms, resulting in early genetic alterations. (b) Scheme of step-wise dedifferentiation of follicular cell-derived thyroid cancer along with genetic alterations.

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Biomarkers, also known as *molecular markers, biological markers*, or *tumor markers* have become useful not only for detecting thyroid cancer early, but also for detecting recurrent and persistent disease and for predicting the effectiveness of surgical removal, radioiodine ablation, and chemotherapy since the past 40 years, and they include genetic mutations and molecular changes. Nowadays, high-throughput genomic and proteomic assays are being used to identify a multitude of biomarker signature for each tumor type at any given stage [39, 40]. These biomarkers are discussed in detail as under.

5.1. Serum-based biomarkers

Serum biomarkers represent the first generation of thyroid biomarkers. Ideally, a serum biomarker is one that is highly sensitive and specific, can establish diagnostic certainty and can be easily measured.

5.1.1. Calcitonin

Para follicular C cells secrete calcitonin, which is a serum-based marker for MTC [41]. Overall, calcitonin is more sensitive for documenting recurrent tumor but CEA levels are better predictors of tumor aggressiveness. *RET* mutations have replaced calcitonin to a greater because it is more sensitive and specific [42].

5.1.2. Thyroglobulin

Tg is a valuable serum marker for detecting recurrent or persistent well-differentiated thyroid cancer of follicular cell origin, as there should be no Tg present after a total thyroidectomy unless residual thyroid tissue is present. More recently, molecular studies using reverse transcriptase-polymerase chain reaction (RT-PCR) have been used to measure tissue-tumor-specific messenger RNA levels of Tg in the circulation [43].

5.2. Mutation-based biomarkers

Genetic alterations in thyroid tumors can be divided into two categories: *inheritable (germline) mutations and sporadic (somatic) mutations*. Investigations into the inheritable and sporadic mutations in thyroid cancer have proceeded in parallel with one another. The single known inheritable gene mutation associated with thyroid cancer is a point mutation in the *RET* proto-oncogene that causes medullary thyroid cancer [44]. The first sporadic mutation identified in thyroid cancer was described in 1987 and involved a genetic defect in the *RAS* protein family [45] followed by somatic *RET/PTC* translocations in 1990 and *P53/NTRK1* mutations in 1992. In the year 2000, *PAX8/PPARgamma* translocations were found in follicular thyroid cancers [46] followed by the discovery of *BRAF* mutations, first in melanoma, then in PTC in 2003 [47]. The mutation-based biomarkers are discussed below in detail.

5.2.1. Chromosomal rearrangements

RET/PTC is a chromosomal rearrangement found in PTC. These chimeric genes contain the portion of *RET* encoding intact tyrosine kinase domain fused to an active promoter of another

gene that drives the expression and ligand-independent dimerization of the *RET/PTC* protein, leading tumorigenesis in thyroid cells [48]. *RET/PTC1* and *RET/PTC3* are the most common rearrangement types in which *RET* is fused to either *CCDC6* (also known as H4) or *NCOA4* (also known as *ELE1* or *RFG*), respectively [49]. Both of these rearrangement types are paracentric, intrachromosomal inversions. *RET/PTC2* and nine more recently discovered types of *RET/PTC* rearrangements are all interchromosomal rearrangements formed by *RET* fusion to genes located on different chromosomes [50]. *RET/PTC* rearrangement occurs in 10–20% of PTC. Thyroid adenomas and other benign nodules and nonneoplastic thyroid lesions have 10–45% of *RET/PTC* rearrangements [51]. Chromosomal rearrangements involving another receptor tyrosine kinase gene, *NTRK1* have been reported to occur in up to 10–15% of PTC in some series of patients although the prevalence of this rearrangement in papillary carcinomas from many geographical areas is probably <2–5% [52]. *PAX8/PPARγ* rearrangement leads to the fusion between a portion of the paired-box gene 8 gene (*PAX8)* and peroxisome proliferator-activated receptor gamma gene (*PPARγ*). The fusion oncoprotein contributes to malignant transformation by targeting several cellular pathways. The *PPARγ* rearrangements are found in follicular thyroid adenomas (0–31%) and follicular thyroid carcinomas (25–63%) [53].

5.2.2. RET point mutations (familial medullary thyroid cancer)

The *RET* gene encodes the *RET* receptor expressed in neuroendocrine and neural cells. The nucleotide sequence of the *RET* gene was determined and in 1989 and was mapped to chromosome 10q11.2 [54, 55]. In 1993, the specific germline mutations of the *RET* gene were found to develop MTC [56]. Point mutations of the *RET* gene that causes MTC result in a gain of function of the *RET* receptor. The hereditary *RET* point mutations are the most specific biomarkers in clinical use today for diagnosing patients who will develop MTC. No other currently used thyroid cancer biomarker is as sensitive or specific.

5.2.3. RAS mutations

The beginning of *RAS* research can be traced back to 1964 when Jennifer Harvey observed that a preparation of a murine leukemia virus, taken from a leukemic rat, induced sarcomas in newborn rodents [57]. The nucleotide sequences of the v-h-ras and v-k-ras oncogenes were not published until the autumn of 1982, a time when the excitement in the *RAS* field was shifting towards the recently isolated human oncogenes. By 1983, a new human transforming gene was identified and found to be a third member of the *RAS* gene family. This gene was designated *NRAS* [58]

5.2.3.1. RAS signaling

RAS proteins are signal switch molecules that regulate cell fates by coupling receptor activation to downstream effector pathways that control diverse cellular responses including proliferation, differentiation, and survival [59]. Human cancers frequently express mutant RAS proteins, termed 'oncogenic RAS'. RAS proteins are GDP/GTP binding proteins that functions as a molecular switches to mediate downstream signaling from a variety of extracellular stimuli. The RAS proteins are activated when the protein binds GTP and be-

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comes inactive upon GTP hydrolysis to GDP by RAS proteins. The action of RAS proteins is regulated by several guanine-nucleotide exchange factor (GNEFs) and GTPase-activating proteins (GAPS). RAS proteins regulate cellular responses to many extracellular stimuli, including soluble growth factors. GTP-bound RAS can interact productively with more than 20 effectors, including Raf, phosphatidylinositol 3-kinase (PI3K) and Ral guanine nucleotide-dissociation stimulator (RALGDS), to regulate various cellular responses including proliferation, survival, and differentiation [60]. RAS–GTP also binds the catalytic subunit of type I PI3Ks causing translocation of PI3K to the plasma membrane and subsequent activation. PI3K phosphorylates phosphatidyl inositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4, 5-triphosphate, which activates downstream kinases such as Akt [61] (**Figure 4**).

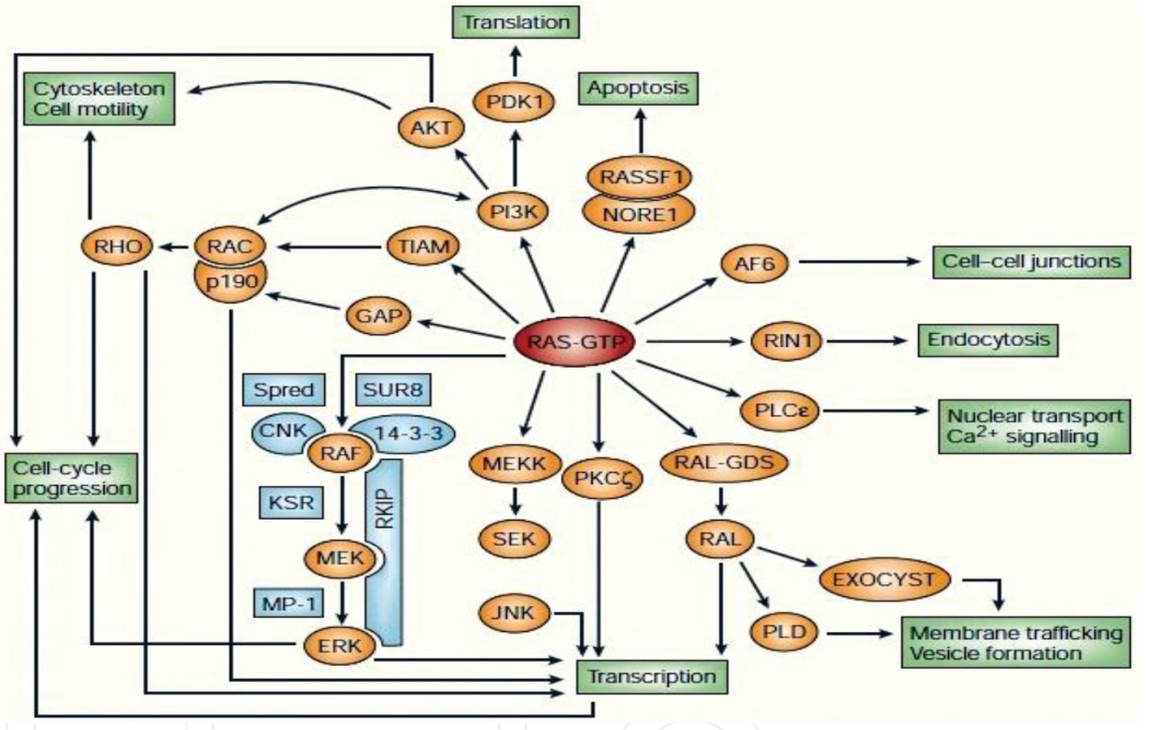

Figure 4. Overview of known RAS effectors and their corresponding biological responses. Active RAS-GTP induces a wide variety of cellular processes, such as transcription, translation, cell-cycle progression, apoptosis or cell survival, through direct interaction with various effectors. GAP proteins also interact with RAS-GTP and might also act as effectors. Modulators of some of these pathways are also indicated. The blue boxes represent adaptor complexes.

5.2.3.2. Oncogenic RAS mutations and abnormal signaling

Somatic missense *RAS* mutations found in cancer cells involve amino acid substitutions at positions 12, 13, and 61 impairing the intrinsic GTPase activity and conferring resistance to GAPs, thereby causing active, GTP-bound conformation to accumulate [62]. Glutamine 61 is essential for GTP hydrolysis, and substituting any amino acid at this position except glutamic acid blocks hydrolysis. Replacing glycine 12 of *RAS* with any other amino acid except proline also biochemically activates RAS. Substituting proline for glycine 12 renders RAS resistant to

GAPs but has increased intrinsic GTP hydrolysis. Consistent with this idea, the transforming potential of HRAS proteins with different codon 61 substitutions is inversely related to intrinsic GTPase activities [63]. Oncogenic RAS proteins deregulate downstream effector pathways to confer the abnormal functional properties of cancer cells: deregulated cell growth, survival, and differentiation.

5.2.3.3. Role of oncogenic RAS gene in thyroid cancer

Activating *RAS* mutations occur in ~30% of human cancers. Activated oncogenes of the *RAS* family have been identified in a wide range of solid and hematological malignancies. Mutations that cause activation of the *RAS* proto-oncogene have been well defined, and several groups have studied the occurrence of different mutations in thyroid neoplasia. The *RAS* mutations generally occur in up to 20–50% of thyroid neoplasms. However, the prevalence of mutations in specific histological classes varies widely. In papillary carcinomas, *RAS* mutations are relatively infrequent, as they occur in 10–20% of tumors. In FTC, *RAS* mutations are found in 40–50% of tumors and may also correlate with tumor dedifferentiation and less favorable prognosis [64]. RAS mutations are found in 20–40% of poorly differentiated and anaplastic carcinomas, 20–40% of benign follicular adenomas [65]. *RAS* mutations may predispose well-differentiated cancers to de differentiation and anaplastic transformation. Because *RAS* mutations are found in the entire spectrum of thyroid cancers, and with increasing frequency as tumors become more undifferentiated, *RAS* mutations have been suggested to be a biomarker for a more aggressive form of thyroid cancer [64]. In Thyroid cancer, *NRAS* codon 61 and *HRAS* codon 61 mutations are most common.

5.2.4. BRAF mutations

Point mutations, small in-frame deletions/insertions, and/or chromosomal rearrangement are the events by which BRAF can be activated. The most common BRAF activation is due to a point mutation involving substitution of thymine by adenine at nucleotide position 1799, resulting in a valine-to-glutamate replacement at residue 600 [66]. This *BRAF* V600E mutation constitutes 98–99% of all *BRAF* mutations found in thyroid cancer. Lys601Glu point mutation and small, in-frame insertions or deletions and *AKAP9*/*BRAF* rearrangement are other alterations in *BRAF* [67]. The *BRAF* V600E mutation is found in 40–45% PTCs. The mutation also occurs in 20–40% of poorly differentiated thyroid carcinomas and 30–40% of ATCs [68].

ARAF, BRAF, and *CRAF* are three *RAF* paralogs. These are downstream molecules of the membrane-bound RAS [69]. RAS stimulates RAF activation, which in turn activates MEK and ERK. ERK regulates cell proliferation, differentiation, senescence, and apoptosis. This pathway is hyper-activated in 30% of cancers with activating mutations in *RAS* occurring in approximately 15–30% of cancers, and recent data have shown that *BRAF* is mutated in about 7% of cancers [70], identifying it as another important oncogene on this pathway. The *BRAF* gene is located on the long (q) arm of chromosome 7 at position 34. More precisely, the *BRAF* gene is located from base pair 140,433,811 to base pair 140,624,563 on chromosome 7.

5.2.4.1. BRAF mutations from A to Z

BRAF mutations are found in 27–70% of malignant melanomas, 36–53% of PTC, 5–22% of colorectal cancers, and <30% of serous ovarian cancer, but they also occur at a low frequency of 1–3% in a wide variety of other cancers [70, 71]. There are more than 40 mutations identified in the *BRAF* gene so far, among which *BRAF* V600E mutation accounts for more than 90% [72, 73]. A few other activated *BRAF* mutants are only rarely found in thyroid cancer, such as the *BRAF* K601E, *AKAP9-BRAF* [74], *BRAF* V599ins [75], K601del, and a recently characterized novel *BRAF* mutant, V600D, FGLAT 601–605ins, resulting from an insertion of 18 nucleotides at nucleotide T1799 of the *BRAF* gene [76].

5.2.4.2. BRAF mutation in thyroid cancer

Although there are lots of alterations in *BRAF* gene in thyroid cancer, the most important mutation found in TC is *BRAF* V600E. This mutation is exclusive to PTC and PTC-derived ATC (44% and 24%, respectively), as it does not occur in any other type of TC.

5.2.4.2.1. Association of BRAF mutation with high-risk clinicopathological characteristics of PTC

Many studies have investigated the relationship of *BRAF* mutation with clinicopathological characteristics of PTC. Although the results are not entirely consistent, most of the studies from various ethnic and geographical backgrounds demonstrate a significant association of BRAF mutation with one or more conventional high-risk clinicopathological characteristics of PTC [77]. Among the various clinicopathological risk factors, extrathyroidal invasion, lymph node metastasis, and advanced clinicopathological stages III and IV most reliably predict thyroid cancer progression, recurrence, aggressiveness, and ultimately, higher morbidity and mortality [78]. Interestingly, among the various clinicopathological characteristics of PTC, many studies have found that *BRAF* mutation is also most commonly associated with these three risk predictors. This suggests that *BRAF* mutation may play a role in promoting the progression of PTC to ATC. Thus, BRAF mutation is a driving force behind the aggressive pathological characteristics of PTC and predicts a poorer prognosis for patients with PTC

5.2.4.2.2. Association of BRAF mutation with recurrence of PTC and loss of radioiodine avidity in recurrent tumors

Many studies have investigated the predictive value of the *BRAF* mutation for PTC recurrence and have shown the association of *BRAF* mutation in the primary PTC with loss of radioiodine avidity in the recurrent tumors [79].

5.2.4.2.3. Molecular bases for BRAF mutation-promoted invasiveness and progression of PTC

The oncogenic strength of *BRAF* mutation and the molecular events coupled to them in the cell cause genetic instability [80]. *BRAF* mutation has a close association with aberrant methylation of several important tumor suppressor genes in PTC including tissue inhibitor of matrix metalloproteinase-3 (*TIMP3*), death-associated protein kinase (*DAPK*), *SLC5A8*, and retinoic acid receptor 2 (*RAR2*) [81] which can further promote invasiveness and progression

of PTC. Interestingly, a recent study demonstrated overexpression of VEGF in association with *BRAF* mutation in PTC [82]. Therefore, adding to the mutation-induced progression and invasiveness of PTC, the authors also showed that *BRAF* V600E promoted activation of the nuclear transcription factor NF kappaB-coupled signaling, which in turn promoted matrigel invasion of thyroid cancer cells. The efficacy of radioiodine treatment for thyroid cancer depends on the integrity of the iodide-metabolizing system of the thyroid cell [83]. Interestingly, *BRAF* mutation was found to be associated with decreased expression of thyroperoxidase (TPO) [84], Na^+/I^- symporter (NIS) [85], Tg [86], and pendrin [87] in primary or recurrent PTC tumors. Conditional expression of *BRAF* V600E in rat thyroid cell lines led to silencing of all these thyroid-specific iodide metabolizing genes [88]. Methylation was shown to be a mechanism mediating the silencing of some of these thyroid genes.

5.2.4.2.4. Testing of BRAF mutation as new dimension to risk stratification and clinical management of PTC

BRAF mutation may represent a novel and useful prognostic molecular marker for PTC. Like several conventional clinicopathological factors, particularly extrathyroidal invasion, lymph node metastasis, and diseases stages III and IV. *BRAF* mutation similarly has a high predictive value for PTC recurrence [85]. This novel prognostic factor may assist in deciding how aggressive the initial treatment of the patient should be and in deciding how vigilantly and aggressively patients should be managed after the initial treatment. PTC patients with *BRAF* mutation may need to be more closely monitored by a more liberal battery of diagnostic tests, such as more aggressive use of imaging methods.

5.2.5. P53 inactivation

P53 is known as "*policeman of the genome*" [89]. Alterations in the *P53* tumor suppressor gene by inactivating point mutations, usually involving exons 5–8, or by deletion result in progressive genome destabilization, additional mutations, and propagation of malignant clones. Among thyroid tumors, *P53* mutations are generally restricted to poorly differentiated thyroid cancer (PDTC) and ATC. Point mutations of *P53* occur in approximately 60% of ATC and in 25% of PDTC [90]. Because of their high incidence in undifferentiated thyroid cancer, the presence of *P53* mutations may be predictive of a highly aggressive thyroid cancer.

5.3. DNA mutation panels

Mutations of the *RET*/*RAS*/*BRAF*/*MAPK* pathway gladiators are responsible for more than 70% of PTCs and 80% of FTCs, but the sensitivity and specificity of these mutations are too low to be clinically relevant. But, because almost 70–80% of thyroid cancers should have at least one of these mutations, a panel of all the mutations may be able to improve the diagnostic accuracy of thyroid tumor FNA cytology. Signatures from gene expression profiles will eventually be used to construct new DNA mutation panels for FNA-based diagnosis of thyroid nodules [91].

5.4. Epigenetic biomarkers

Currently, epigenetic refers to the study of heritable changes in gene expression that occurs without any alteration in the primary DNA sequence [92]. Epigenetic information that fulfills the requirement of heritability can be classified into three distinct types: *DNA methylation*, *histone modifications*, and *noncoding RNAs*. In thyroid cancer, DNA methylation, histone modifications, and microRNA silencing have all been studied, but there is minimal data on nucleosome positioning.

Aberrant methylation, or hypermethylation, of tumor suppressor genes has been identified in many human tumors including thyroid tumors [93]. Hypermethylation of multiple genes has been identified in association with the PIK3/AKT pathway in FTC and of the MAPK pathway in PTC. Hypermethylation has also been identified in benign thyroid tumors, though to a lesser extent than in thyroid carcinomas. A close association between *BRAF* mutation and aberrant methylation of several tumor-suppressor genes in PTC has been reported [81]. Aberrant methylation also involves thyroid-specific genes such as the *NIS*, the promoter of the TSH receptor, the genes for the putative thyroid follicular cell apical iodide transport (*pendrin* and *SCL5A8*) [93]. Suppression of these thyroid iodide-metabolizing molecules results in the loss of cancer cells ability to concentrate iodine, rendering tumors insensitive to radioiodine therapy.

5.4.1. TSHR function and signaling

TSH is the main regulator of thyroid gland growth and development. Binding of TSH to TSHR stimulates thyroid epithelial cell proliferation and regulates the expression of differentiation markers such as Tg, TPO, and the NIS, necessary for the synthesis of thyroid hormones. Two G protein-dependent pathways are activated by TSHR: (i) Gαs-adenylate-cAMP activates protein kinase A (PKA)—phosphorylates the transcription factor CREB, thereby increasing its transcriptional activity and (ii) Gαq-phospholipase C–releasing inositoltriphosphate (IP3) and diacylglycerol (DAG)—activates protein kinase C, which promotes proliferation via the *RAF/MEK/ERK* pathway. Complex cross-talk occurs between these pathways and other signaling pathways including the PI3/Akt, PKC/NFkB, and JAK/STAT pathways [94, 95].

5.4.2. TSHR alterations related to thyroid cancer

Excesses or defaults in TSHR activity may play a role in thyroid disease and cancer. Both can be achieved by a number of mechanisms including mutations in critical domains, improper epigenetic marking of the gene, or incorrect transcriptional regulation.

5.4.3. Altered levels of TSHR expression

Quantitative analysis of promoter hypermethylation in thyroid cancer has involved *RASSF1A, TSHR, RARβ2, DAPK, S100, p16, CDH1, CALCA, TIMP3, TGF-β*, and *GSTpi* [81]. The *TSHR* gene promoter is frequently hypermethylated in thyroid carcinoma, with preferential methylation in undifferentiated carcinoma. In contrast, *TSHR* gene promoter is unmethylated in the normal thyroid and in benign tumors (thyroid adenoma). TSHR stimulates several key

steps in thyrocyte concentration of iodine, including uptake by NIS and oxidation before incorporation into Tg by thyroid peroxidase [96]. Promoter hypermethylation resulting in decreased expression of *TSHR* and *NIS* may result in a decreased ability to concentrate iodine, rendering ablative doses of 131I ineffective. Promoter hypermethylation of *TSHR* is reported in 34–59% of patients with PTC [97]. NIS expression and iodide uptake requires functional TSHR. Low or absent TSHR expression correlates with worse prognosis in thyroid carcinomas [98].

5.4.4. BRAF mutational status and silencing of TSHR gene

It is thought that the loss of responsiveness to 131I is because of the loss of function of iodine-metabolizing proteins, such as NIS and TSHR. Tumor cells harboring *BRAF* V600E mutation have decreased NIS and TSHR gene expression compared with similar cells without the mutation. Several recent in vitro and in vivo mouse studies have demonstrated that BRAF inhibition with small-molecule MAPK pathway inhibitors restores the expression of iodine-metabolizing proteins and increases susceptibility to radioiodine ablation [99]. *BRAF* mutations are associated with decreased expression of mRNAs for the NIS and the TSH receptors that are considered markers of thyroid differentiation [100] (**Figure 5**).

The molecular mechanism involved in this V600E *BRAF*-induced silencing of thyroid genes is also unclear. Liu et al. showed the restorability of the expression of several key thyroid iodide-metabolizing genes by suppressing BRAF/MEK/MAP kinase pathway in thyroid cells expressing the V600E *BRAF* mutant. Using TSHR gene as a model, they showed that the effect

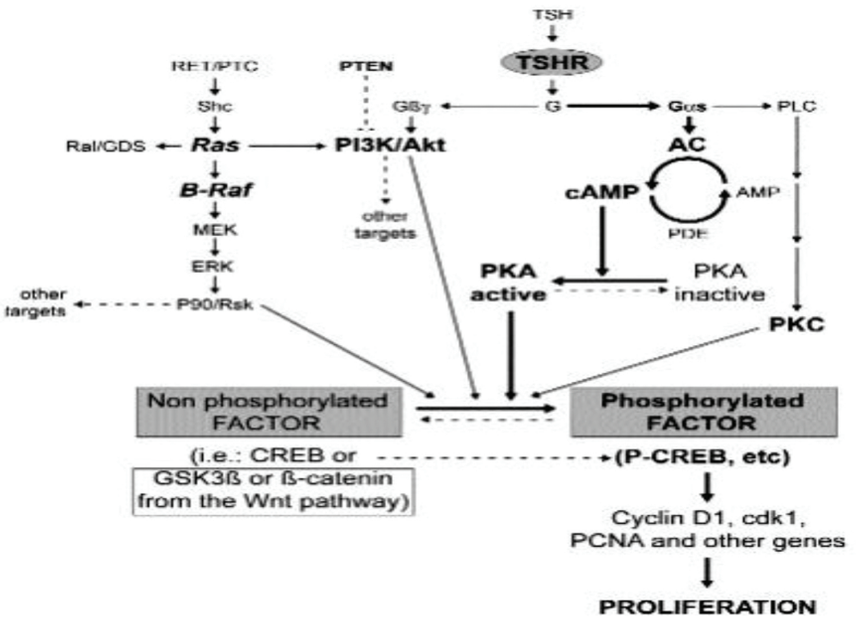

Figure 5. Classical TSHR signaling pathway and the framing network in thyrocyte proliferation. The bold arrows represent the classical TSHR signaling pathway towards proliferation. Normal arrows integrate cross talking molecules from other signaling pathways. Dashed lines represent other targets that may or may not be related to this pathway. Commonly altered molecules in thyroid cancer that may alter the integrity of the signaling network are enclosed in a square. Examples of integration between the classical TSHR/PKA and the MAPK/ERK, PI3K/Akt and Wnt/β-catenin pathways are provided.

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of the *BRAF/MEK/MAP* kinase pathway on thyroid gene expression occurred through alteration of gene promoter activity, which may involve methylation [101].

5.5. Genomics

It includes the mapping and sequencing of the genome, as well as the analysis of the information gained from mapping and sequencing in the context of their biological significance and biomedical application. cDNA microarrays, oligonucleotide arrays, and serial analysis of gene expression (SAGE) are the various gene expression profiling technologies currently in use [102] which allow the study and comparison of the expression of thousands of genes simultaneously in varying conditions and will someday lead to the development of DNA signatures unique to each patient leading to patient specific treatment. In 2001, the first gene expression profile in thyroid cancer was done [103].

5.6. Proteomics

Proteomics is defined as the study of protein structure and function. The term was first introduced as an analogy to "genomics", but in this case referring to the entire protein spectrum [104]. Several immunohistochemical markers representing different components of the cell, such as the membrane, the cytoplasm, or the nucleus, have been studied in thyroid neoplasms [105]. The proteomic information also takes into account post-translational changes that are not detected at the mRNA level, as well as protein expression. The advantage of proteomics is the ability to detect biomarkers leaked into circulation from the patient's serum or plasma. Proteomics combines multidimensional separation systems based on mass spectrometry analysis and protein chip technology to detect complex mixture of proteins and peptides from either tissue or serum with high sensitivity and specificity [106]. The first study that established a proteomic profile of benign and malignant human thyroid tissue was reported in 2002 [107].

5.6.1. BRAF protein overexpression in thyroid cancer

The BRAF copy number gain, which results from either numerical changes of chromosome 7 or gene amplification, occurs in a significant portion of benign and malignant follicular thyroid tumors, including those of conventional and oncocytic types. This abnormality is associated with overexpression of BRAF protein and did not coincide with the presence of other mutations leading to activation of the MAPK pathway, suggesting that BRAF copy number gain may represent another mechanism of BRAF activation in thyroid tumors. It has been known for a long time that clonal numerical changes of chromosome 7 are common in benign and malignant thyroid tumors, and most of them are chromosome gains, particularly trisomy 7 [108]. Although gains of chromosome 7 lead to the increase in copy number of many genes located on this chromosome, data suggest that BRAF may represent an important target for the selection and clonal progression. The numerical changes of BRAF include gains of one to three extra copies of the gene and result in the modest overexpression (near to double) of the protein. This increased protein expression leads to additional stimulation of the MAPK pathway, although significantly lower as compared to more than 400-fold increase of BRAF kinase activity imposed by V600E point mutation [109]. A study by Kondo et al. revealed focal

expression of wild-type BRAF in nonneoplastic thyroids and diffuse expression in benign adenomas and well-differentiated carcinomas regardless of their *BRAF* gene mutational status. Increased expression of wild-type *BRAF* may play important roles in the proliferation of transformed follicular cells [1].

6. Molecular analysis of *BRAF* and *RAS* genes

The study was aimed and designed to analyze the mutations, if any, in the coding exons (1 and 2) of RAS gene family (*NRAS, HRAS* and *KRAS*) and exon 15 of *BRAF* gene along with the analysis of BRAF protein expression and to establish the correlation of *RAS* and *BRAF* gene mutation and BRAF expression with clinicopathological variables of thyroid cancer patients. A total of 60 consecutive thyroid tumors and their adjacent normal tissues surgically resected either by total thyroidectomy/hemi-thyroidectomy or lobectomy over a period of 3 years were included in the study for sequence analysis of *RAS* gene family (*HRAS, NRAS*, and *KRAS*) and *BRAF* gene. By histopathological conformation, all the resected samples were established as thyroid cancer. Majority of the patients had attended the hospital with a clinical presentation of a lump or nodule. In this study, 80% (48 of 60) of cases were females and 20% (12 of 60) were males with a male: female ratio of 1:4. The cases in the age group of <45 were 60% (36 of 60) and exceeded than ≥45 years which were 40% (24 of 60). Only 10% (6 of 60) of patients were smokers who were all males and 90% (46 of 60) were nonsmokers. Benign thyroid diseases were found in 80% (48 of 60) patients. Tumor samples were histologically confirmed as differentiated thyroid carcinomas [PTC—70% (42 of 60) and FTC—13.4% (8 of 60), respectively] except few cases of MTC—6.6% (04 of 60) and Hürthle cell cancer—10% (6 of 60). Well-differentiated cancer grade was present in 95% (57 of 60) thyroid cancer patients. The clinicoepidemiological and pathological characteristics of these patients are listed in **Table 3**.

DNA isolated from the samples (tumor tissues and corresponding normal tissues) (**Figure 6**) was subjected to PCR to amplify the hot spot coding exon 15 of *BRAF* gene. Besides these, six other coding exons of *RAS* family of genes were amplified (1 and 2 exons of *HRAS, NRAS* and *KRAS*). The representative pictures of each exon of both the genes are given in **Figure 7**. After PCR amplification, the PCR products were subjected to DNA sequence analysis.

To identify the sequence variations, the electrophoregram obtained after sequencing of the PCR products was compared manually with the reference sequence of the *BRAF* and *RAS* genes deposited in the NCBI gene bank database. In addition, the electrophoregrams of both the genes were compared with the corresponding reference sequence of *BRAF* and *RAS* gene by aligning in "Cluster X software" to find somatic aberrations like insertions, deletions, or substitutions.

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Variable	Parameter	Cases (n = 60)	
		n	% n
Sex	Female	48	80
	Male	12	20
Age, years	<45	36	60
	≥45	24	40
Dwelling	Rural	51	85
	Urban	09	15
Smoking status	Nonsmoker	54	90
	Smoker	06	10
Benign thyroid disease	Yes	48	80
	No	12	20
TSH levels	Elevated	25	41.6
	Normal	35	58.4
Histological types	Papillary	42	70
	Follicular	08	13.4
	Others	10	16.6
Grade	Well differentiated	57	95
	Poorly differentiated	03	05
Stage, <45 years	Stage I	34	56.6
	Stage II	02	3.4
Stage, ≥45 years	Stages I and II	15	25
	Stage III and above	09	15
Lymph node metastasis	Yes	15	25
	No	45	75
Vascular/capsular invasion	Yes	26	43
	No	34	67

TSH = Thyroid-stimulating hormone, n = Number.

Table 3. Clinicoepidemiological and clinicopathological variables of thyroid cancer patients used for mutational analysis in our center (SKIMS, India).

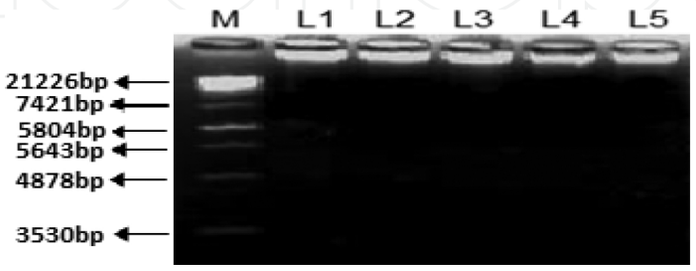

Figure 6. 1% Agarose gel electrophoresis of DNA isolated from blood, tumor tissue, and adjacent normal tissue of thyroid cancer patient. Lane M consists of lambda DNA-EcoRI digest. Lanes 1–3: DNA derived from thyroid tumor tissue. Lane 4: DNA derived from adjacent normal tissue. Lane 5: DNA derived from blood of thyroid cancer patient.

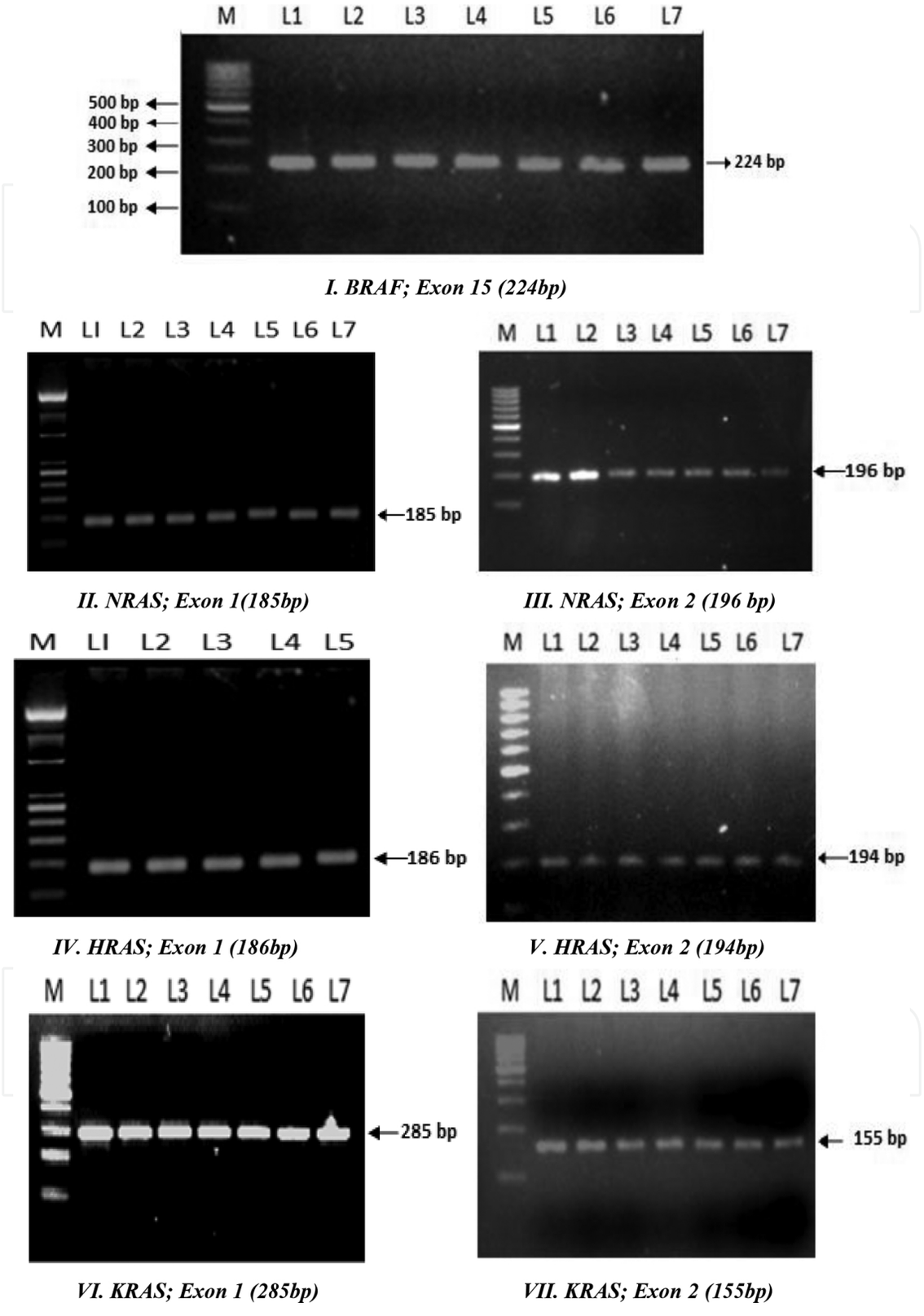

Figure 7. PCR amplification of different exons of BRAF and RAS genes. Lane M: molecular size marker 100 bp. Lanes 1–5, 6 and 7: amplified product from DNA of patient samples.

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6.1. Mutational spectrum of BRAF gene

Total mutations of *BRAF* in this study were found to be 25% (15 of 60). All of them were transversions (T > A) at nucleotide position 1799 in exon 15. This mutation affects codon 600 of *BRAF* gene. This V600E mutation was further confirmed by reverse sequence of the same samples (**Figures 8** and **9**). The matched constitutional DNA contained the wild-type sequence in every case, demonstrating the somatic nature of these mutations in thyroid cancer.

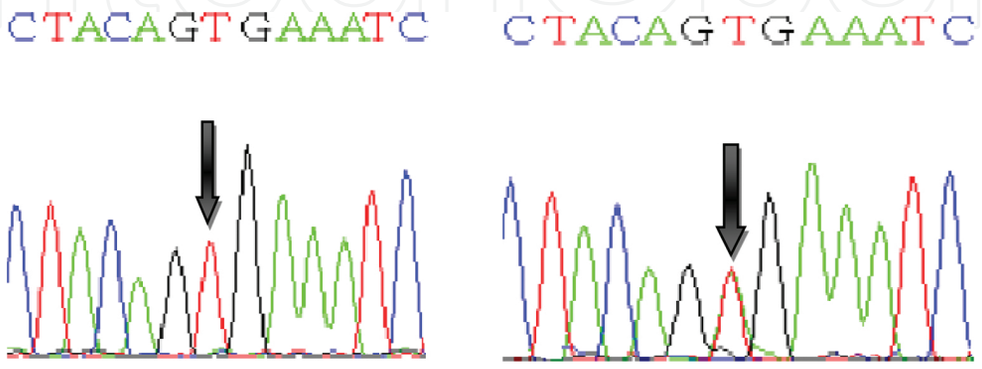

Figure 8. Partial electropherograms (forward) of the adjacent normal (left) and mutants (right) in exon 15 of the BRAF gene codon 600 (TGA→CGA).

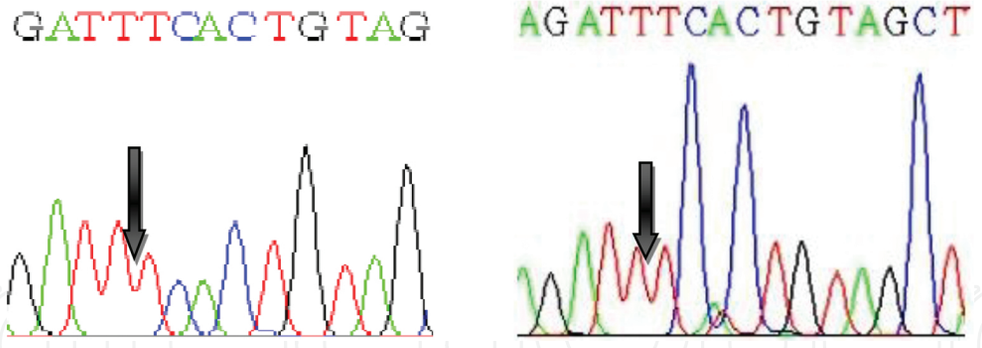

Figure 9. Partial electropherograms (reverse) of the adjacent normal (left) and mutants (right) in exon 15 of the BRAF gene codon 600 (TGA→CGA).

Among 25% (15/60) mutations of *BRAF* gene found in this study, 40% (10 of 25) of cases having elevated TSH levels were harboring mutation compared to 14.2% (05 of 35) cases having normal TSH levels and this difference showed a strong statistical significance ($P < 0.05$) (**Table 4**). Among the various histological types of thyroid cancer, mutations were restricted only to PTC. So, 35.7% (15 of 42) of PTC patients were having mutation in codon 600 of *BRAF* gene compared to follicular and other types of thyroid cancer which did not contain any mutation and this difference in mutation frequency between different histological types of tumors was statistically significant ($P < 0.05$). All the mutations were found in well-differen-

tiated thyroid carcinomas (26.3% – 15 of 57) when compared to poorly differentiated thyroid carcinomas ($P < 0.05$). In thyroid cancer patients having <45 years of age, 23.5% (8 of 34) of patients with stage I disease had mutation compared to 100% (02 of 02) in stage II patients. Similarly, thyroid cancer patients having ≥45 years of age, 33.3% (05 of 15) of patients with stage I disease have mutation compared to stage II patients who were free from mutation ($P < 0.05$). In this study, 34.6% (9 of 26) patients having vascular and capsular invasion were having mutation compared to only 17.6% (06 of 34) of mutation positive patients free from invasion ($P < 0.05$). No significant association of this mutation was found in this report with any other clinicoepidemiological characteristics of thyroid cancer patients (**Table 4**) [110].

Characteristics	**Cases (n = 60)**		**Mutants n = 15 (25%)**		**Wild type n = 45 (75%)**		**P-Value**
	n	**% n**	**n**	**% n**	**n**	**% n**	
Sex							
Female	48	80	12	25	36	75	>0.05
Male	12	20	03	25	09	75	
Age, years							
<45	36	60	10	27.7	26	72.3	>0.05
≥45	24	40	05	20.8	19	79.2	
Dwelling							
Rural	51	85	13	25.4	38	74.6	>0.05
Urban	09	15	02	22.2	07	77.8	
Smoking status							
Nonsmoker	54	90	13	24.1	41	75.9	>0.05
Smoker	06	10	02	33.3	04	66.7	
Benign thyroid disease							
Yes	48	80	13	27.1	35	72.9	>0.05
No	12	20	02	16.7	10	83.3	
TSH levels							
Elevated	25	41.6	10	40	15	60	**<0.05**
Normal	35	58.4	05	14.2	30	85.8	
Histological types							
Papillary	42	70	15	35.7	27	64.3	**<0.05**
Follicular	08	13.4	00	00	08	100	
Others	10	16.6	00	00	10	100	
Grade							
Well differentiated	57	95	15	26.3	42	73.7	**<0.05**
Poorly differentiated	03	05	00	00	03	100	

Characteristics	Cases (n = 60)		Mutants n = 15 (25%)		Wild type n = 45 (75%)		P-Value
	n	% n	n	% n	n	% n	
Stage, <45 years							
Stage I	34	56.6	08	23.5	26	76.5	**<0.05**
Stage II	02	3.4	02	100	00	00	
Stage, ≥45 years							
Stages I and II	15	25	05	33.3	10	66.7	**<0.05**
Stage III and above	09	15	00	00	09	100	
Lymph node metastasis							
Yes	15	25	07	46.6	08	53.4	**<0.05**
No	45	75	08	17.7	37	82.3	
Vascular/capsular invasion							
Yes	26	43	09	34.6	17	65.4	>0.05
No	34	67	06	17.6	28	82.4	

TSH = Thyroid-stimulating hormone, n = number.

Table 4. Clinicoepidemiological and clinicopathological variables of thyroid cancer patients versus the mutant phenotypes of the BRAF gene.

The substitution of the negatively charged glutamic acid for an uncharged valine at position 600 may mimic the normal physiological phosphorylation of T599 and S602 resulting in a constitutively activated BRAF kinase [71] and stimulating BRAF activity up to 700-fold [111]. Studies along with an updated meta-analysis continue to show a strong relationship of *BRAF* mutation with aggressive clinicopathological characteristics of PTC [112, 113]. In conclusion, our study shows that the *BRAF* mutations characterize the aggressive pathway of thyroid tumorigenesis.

6.2. Mutational spectrum of RAS genes

Exons 1 and 2 each of *NRAS*, *HRAS*, and *KRAS* genes were screened for mutations in 60 tissue samples of thyroid cancer cases. Total six exons of *RAS* gene family were screened for mutations especially in codons 12, 13, and 61. No mutations were observed in any of the six exons studied, particularly in codons 12, 13, and 61 (**Figure 10**). Studies on a variety of tumors have demonstrated some "hot spots" in *RAS* gene family that are susceptible to point mutations. Many studies have detected different types of *RAS* mutations in human thyroid tumors [114, 115], but *RAS* gene family members have not been screened for mutation in the same sample series in thyroid tumors in Kashmiri patients. Activating *RAS* mutations have been reported to occur in ~30% of human cancers [116]. Our study was limited to screening of two hot spot exons of each *RAS* family of genes but in contrast to most of the studies showed no activating mutations in the thyroid tumors [117, 118]. Furthermore, many studies have

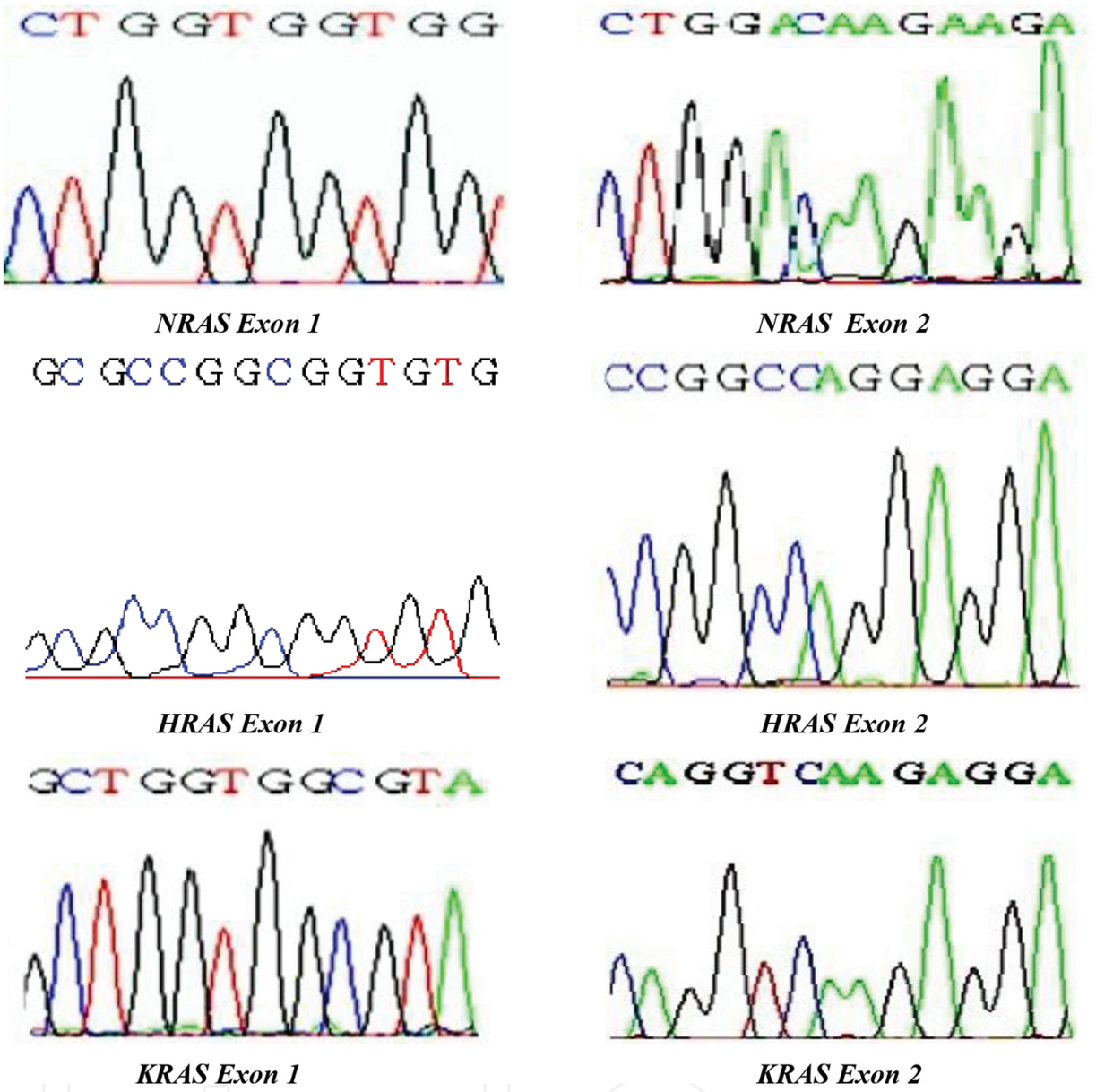

Figure 10. Partial electropherograms (forward) of exons 1 and 2 of the NRAS, HRAS, and KRAS genes.

reported mutual exclusiveness of *BRAF*, *RAS* as well as *RET*/*PTC* rearrangements in papillary thyroid cancers [1, 119]. As PTC is more prevalent in our region *BRAF* mutations predominate; hence, *RAS* mutations were not found in our study due to their mutual exclusiveness. In conclusion, it is evident from our study that although thyroid cancer is highly prevalent in this region, the mutational events for *RAS* genes do not seem to be involved in the thyroid carcinogenesis.

6.3. Polymorphic study of HRAS T81C SNP

DNA sequencing of *HRAS* exon 1 showed frequent T to C substitution in codon 27 of exon 1 at cDNA position 81, which is located in a wobble base position (**Figure 11**). The substitution

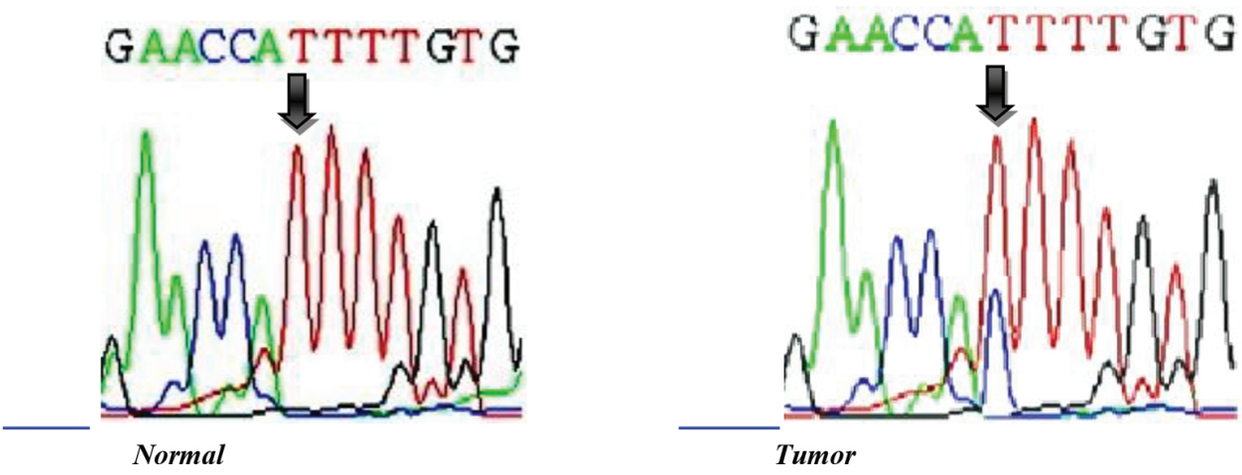

Figure 11. Partial electropherograms (forward) of the adjacent normal (left) and mutants (right) in exon 1 of the HRAS gene codon 27 (CAT→CAC).

(T81C) in codon 27 was found in 16 of 60 (26.6%) tumor tissue samples. *HRAS* 81 T > C substitution was found in 12 of 42 (28.5%) PTC tissues and 04 of 08 (50%) FTC tissues. *HRAS* T81C was frequently observed and was considered to be an informative SNP. Since this polymorphism has been reported only once in thyroid cancer; further, evaluation was imperative, to elucidate the conformity of the results in the backdrop of different ethnic backgrounds; thus, we conducted a case-control polymorphic study of *HRAS* T81C to assess the role of this SNP in thyroid cancer in Kashmiri population (North India). A total of 140 peripheral blood samples from confirmed thyroid cancer patients were collected from the department of Nuclear Medicine, SKIMS over a period of two years. Also 170 blood samples were collected from control subjects who were not having any sort of malignancy from the same hospital and belonging to the same geographical area, ethnic background for polymorphic analysis of *HRAS* T81C SNP. The cases included 19% (26 of 140) males and 81% (114 of 140) female patients (1:4.4), and the controls consisted of 82.4% (140 of 170) males and 17.6% (30 of 170) females. Of the total number of cases, 89% (124 of 140) were nonsmokers and 11% (16 of 140) were smokers. The subjects were considered nonsmokers only if until the day of sample collection they had not consumed tobacco and subjects were considered smokers if they are smoking presently or had quit smoking since last 6 months or less before sample collection. Only 29% (40 of 140) patients were above 45 years of age, and 71% (100 of 140) patients were below 45 years of age. **Table 5** shows demographic information and other parameter of cases and controls. The representative pictures of the amplicons and the RFLP are shown in **Figure 12**. The distribution of *HRAS* T81C allele frequency, its genotypes in cases and controls are shown in **Tables 6** and **7**. Due to the very low frequency of the 'CC' genotype and an increased risk associated with TC and CC genotypes, TC + CC was compared against TT. Frequencies of TT, TC, and CC genotypes among cases were 41.4%, 38.6%, and 20%, while in controls 84.1%, 11.7%, and 4.2%, respectively, with odds ratio (OR) of 7.4; 95% confidence interval (CI) = 4.3–12.7. The cases had a higher frequency of the rare allele (TC + CC) (58.6%) than the controls (15.9%), and this pattern of distribution of rare alleles among two groups

showed statistical significance (P < 0.05). This finding shows an increased risk with TC + CC combination of genotypes against TT genotype. The frequency of mutant C allele was 39.3% in cases and 10% in controls. This observation showed a highly statistical significance of rare allele (C) between cases and controls (P < 0.05) with an O.R (95% C.I) of 5.8 (3.7–8.7). When classified further into groups, our study interestingly found higher percentage of rare allele (TC + CC) in FTC (82%, 18 of 22) compared to PTC (54%, 64 of 118) (P < 0.05). Association of variant allele with other clinicopathological characteristics is given in **Table 7**. While age, dwelling, gender, smoking status, and genotype (TC + CC) were associated with thyroid cancer in odds adjusted univariate analysis, the same parameters were associated with this disease in multivariate logistic regression analysis [120].

Characteristics	Cases n = 140 (%)	Controls n = 170 (%)	χ^2-Value	P-Value
Age group				
<45	100 (71)	60 (35)	40.14	**<0.05**
≥45	40 (29)	110 (65)		
Sex				
Female	114 (81)	30 (17.6)	125.56	**<0.05**
Male	26 (19)	140 (82.4)		
Dwelling				
Rural	112 (80)	50 (29.4)	78.75	**<0.05**
Urban	28 (20)	120 (70.6)		
Smoking				
Never	124 (89)	50(29.4)	109.12	**<0.05**
Ever	16 (11)	120 (70.6)		
Benign thyroid disease				
Yes	84 (60)			
No	56 (40)			
TSH levels				
Elevated	100 (71)			
Normal	40 (29)			
Histological types				
Papillary	118 (84)			
Follicular	22 (16)			
Tumor grade				
WD	134 (96)			
PD	06 (04)			
Stage, <45 years				
Stage I	94 (67)			
Stage II	06 (4.3)			

Characteristics	Cases n = 140 (%)	Controls n = 170 (%)	χ^2-Value	P-Value
Stage, ≥45 years				
Stages I and II	36 (25.7)			
Stage III and above	04 (03)			
Vascular/capsular invasion				
Yes	68 (48.5)			
No	72 (51.5)			
Lymph node metastasis				
Yes	52 (37)			
No	88 (63)			

TSH = thyroid-stimulating hormone, WD = well-differentiated thyroid cancer, PD = poorly differentiated thyroid cancer.

Table 5. Frequency distribution analysis of selected demographic and risk factors in thyroid cancer cases and controls taken for HRAS T81C polymorphic study.

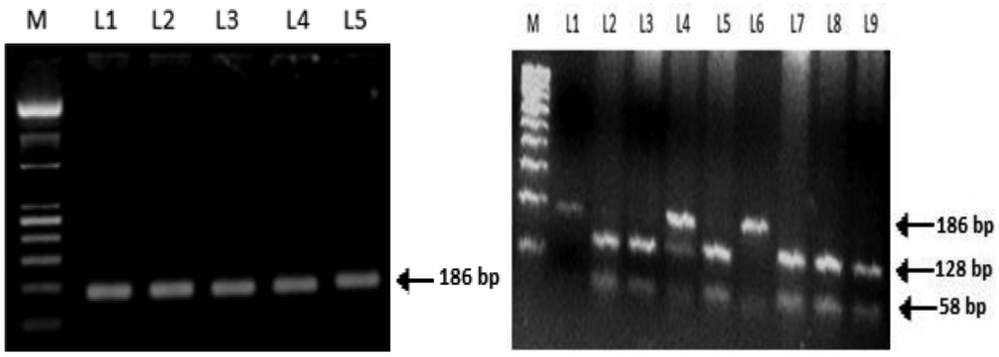

Figure 12. (A) PCR-amplified product of HRAS exon 1 (186 bp). (B): fragment digestion of PCR product by DraIII. TT allele (186 bp) shown in lanes 1 and 6; the TC heterozygous (186 bp, 128 bp and 58 bp) in lane 4; and homozygous CC variant (128 bp and 58 bp) in well 2, 3, 5, 7–9; M = 100 bp ladder.

	Cases n = 140 (%)	Controls n = 170 (%)	OR (95% CI)	P-Value
Genotype				
TT	58 (41.4)	143 (84.1)	6.6 (3.6–12.0)	**<0.05**
TC	54 (38.6)	20 (11.7)	9.8 (4.0–23.6)	**<0.05**
CC	28 (20)	07 (4.2)		
Allele type				
T	170 (60.7)	306 (90)	5.8 (3.7–8.7)	**<0.05**
C	110 (39.3)	34 (10)		

Table 6. Distribution of HRAS T81C genotypes and its allele frequency in cases and controls.

	Cases n (%)	TT	TC + CC	Controls n (%)	TT	TC + CC	OR (95% CI)	Adjusted OR (95% CI)	P-value
Overall genotype	n = 140	58	82	n = 170	143	27	7.4 (4.3–12.7)	7.4 (4.3–12.7)	**<0.05**
Age group									
<45	100 (71)	40	60	60 (35)	49	11	6.7 (3–14.4)	3.9(1.7–9.2)	**<0.05**
≥45	40 (29)	18	22	110 (65)	94	16	7.1 (3.1–15.6)	6.9(2.6–17.7)	**<0.05**
Sex									
Female	114 (81)	50	64	30 (17.6)	26	04	8.3 (2.6–25.3)	7.6(2.0–28.8)	**<0.05**
Male	26 (19)	08	18	140 (82.4)	117	23	11.4(4.3–29.2)	11.5(3.6–36.9)	**<0.05**
Dwelling									
Rural	112 (80)	40	72	50 (29.4)	34	16	3.8 (1.8–7.7)	3.7(1.5–9.1)	**<0.05**
Urban	28 (20)	18	10	120 (70.6)	109	11	5.5 (2.0–14.8)	5.2(1.4–9.1)	**<0.05**
Smoking									
Never	124 (89)	48	76	50(29.4)	33	17	03(1.5–5.9)	3.1(1.3–7.4)	**<0.05**
Ever	16 (11)	10	06	120 (70.6)	110	10	6.6 (1.98–21.7)	7.2(1.2–42.0)	**<0.05**
Benign thyroid disease									
Yes	84 (60)	34	50				1.1 (0.5–2.42)		>0.05
No	56 (40)	24	32						
TSH levels									
Elevated	100 (71)	44	56				0.7 (0.3–1.6)		>0.05
Normal	40 (29)	14	26						
Histological types									
Papillary	118 (84)	54	64				0.26 (0.06–1.0)		**<0.05**
Follicular	22 (16)	04	18						
Tumor									
Grade	134 (96)	56	78				0.7 (0.05–8.6)		>0.05
WD	06 (04)	02	04						
PD									
Stage, < 5 years									
Stage I	94 (67)	38	56				0.7 (0.06–8.7)		>0.05
Stage II	06 (4.3)	02	04						
Stage, ≥45 years									
Stages I and II	36 (25.7)	16	20				1.25(0.15–9.8)		>0.05
Stages III and above	04 (3)	02	02						
Vascular/capsular invasion									
Yes	68 (48.5)	32	36				0.63 (0.32–1.2)		>0.05
No	72 (51.5)	26	46						
Lymph node metastasis									
Yes	52 (37)	22	30				0.9 (0.39–2)		>0.05
No	88 (63)	36	52						

TSH = thyroid-stimulating hormone, WD = well-differentiated thyroid cancer, PD = poorly differentiated thyroid cancer.

Table 7. Association between HRAS T81C phenotypes and clinicopathologic characteristics of thyroid cancer patients.

In thyroid cases, however, we found higher frequency of variant genotypes as compared to other studies conducted on various cancers [121]. Our study revealed a sevenfold increased risk of thyroid cancer in carriers of the variant genotype (TC + CC) in cases. Therefore, our report reveals a significant risk for thyroid cancer, both either when stratified with C allele or in combination of the variant genotypes TC + CC compared with the TT genotype. Consistent with the tissue specificity hypothesis and various studies that had confirmed that the *HRAS* gene plays a more important role in bladder cancer acquired amino acid mutations in the hotspot codons 12, 13, and 61, which prolong the GTP-bound activated state of the *HRAS* product [122]. This polymorphism does not lead to the alteration of RAS protein structure, and it affects the cancer susceptibility possibly through linkage disequilibrium with other potential functional variants of *HRAS*. One of the linkage candidates is a region of variable tandem repeats about 1 kb downstream exon 4, with a possible transcriptional enhancer activity [123]. Another associated polymorphic site is hexanucleotide repeat located about 80 bp upstream of the 5′-end of exon 1 [124]. Yet another report has shown that *HRAS* T81C might be serving as a marker of other polymorphisms in intron D2 of *HRAS* that would act as regulators of IDX inclusion [125]. In conclusion, *HRAS* T81C SNP has been found to moderately increase thyroid cancer risk with variant alleles implicated more in follicular thyroid tumors.

6.4. Analysis of protein expression of BRAF

In this part of study, a total of 60 previously analyzed TC and their adjacent normal tissues were further analyzed for BRAF protein expression. **Table 3** depicts the clinicopathological characteristics of the studied subjects. **Figure 13** shows the representative picture of the extracted proteins run on SDS PAGE. Out of 60 cases of thyroid cancer, 90% (54 of 60) showed overexpression of BRAF protein (**Figure 14**) and the rest 10% (6 of 60) of the cases showed normal protein (BRAF) expression. Overexpression of BRAF protein in males was observed to be 84% (10 of 12) and in females as 91.6% (44 of 48). Among nonsmokers 96.3% (52 of 54) showed overexpression compared to smokers who showed only 33.4% (2 of 6) overexpression in BRAF protein and the difference is statistically significant (P > 0.05). BRAF protein overexpression was found to be in 97.7% (41 of 42) of PTC, 75% (6 of 8) of FTC, and 70% (07 of 10) of medullary/

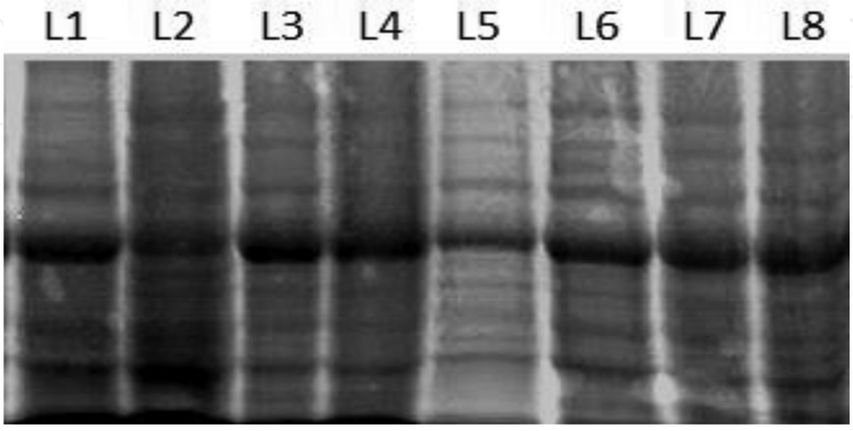

Figure 13. Representative gel picture of 10% SDS-PAGE. In each case 24 μl sample (20μl of the crude protein extract + 4 μl sample buffer) from tumor tissue and adjacent normal was loaded.

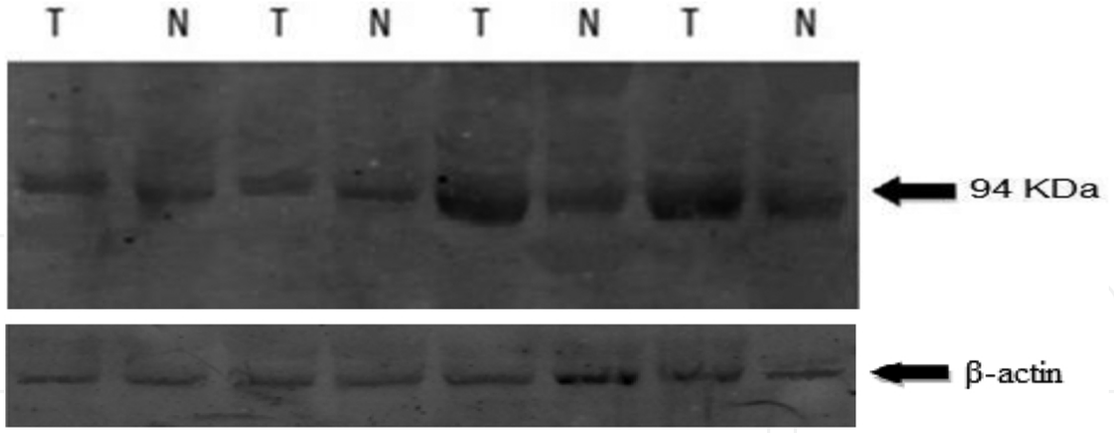

Figure 14. Western blot analysis of BRAF protein in thyroid tumor and adjacent normal tissues. Representative immunoblot showing the expression of BRAF protein in thyroid tumor tissue as compared to their adjacent normals. Extracts from samples were separately run for β-actin protein expression as loading control. Lanes T: protein extracted from tumor tissue. Lanes N: protein extracted from normal tissues. Membrane was probed with a polyclonal antibody specific for BRAF protein.

Hürthle cell carcinomas with a statistically significant association (P > 0.05). When we compared *BRAF* gene mutational status with BRAF protein expression, 86.7% (13 of 15) of *BRAF* mutation positive patients were having overexpression of BRAF protein, whereas 91.2% (41 of 45) of patients having wild-type *BRAF* status were having overexpressed BRAF protein (P > 0.05). No significant association of BRAF overexpression with any other clinicopathological characteristics was found (**Table 8**) [110].

		Normal expression n (%)	**Over expression n (%)**	**OR (95% CI)**	**P-Value**
Clinico pathological variables	**Overall cases n = 60 (%)**	**06 (10%)**	**54 (90%)**	–	–
Sex					
Female	48 (80%)	04 (8.4%)	44 (91.6%)	Reference	>0.05
Male	12 (20%)	02 (16%)	10 (84%)	2.2 (0.35–13.6)	
Age, years					
<45	36 (60%)	02 (5.5%)	34 (94.5%)	Reference	>0.05
≥45	24 (40%)	04 (16%)	20 (84%)	3.4 (0.54–20)	
Dwelling					
Rural	51 (85%)	04 (7.8%)	47 (92.2%)	Reference	>0.05
Urban	09 (15%)	02 (22.2%)	07 (77.8%)	3.3 (0.5–21.4)	
Smoking status					
Nonsmoker	54 (90%)	02 (3.7%)	52 (96.3%)	Reference	**<0.05**

		Normal expression n (%)	Over expression n (%)	OR (95% CI)	P-Value
Smoker	06 (10%)	04 (66.6%)	02 (33.4%)	52 (5.2–468)	
Benign thyroid disease					
Yes	48 (80%)	04 (8.3%)	44 (91.7%)	Reference	>0.05
No	12 (20%)	02 (16.6%)	10 (83.4%)	2.2 (0.35–13.7)	
TSH levels					
Elevated	25 (41.6%)	02 (8%)	23 (92%)	Reference	>0.05
Normal	35 (58.4%)	04 (11.5%)	31 (88.5%)	1.5 (0.24–8.9)	
Histological types					
Papillary	42 (70%)	01 (2.3%)	41 (97.7%)	Reference	**<0.05**
Follicular	08 (13.4%)	02 (25%)	06 (75%)	13.6 (1–174)	
Others	10 (16.6%)	03 (30%)	07 (70%)	17.5 (1.6–192)	
Tumor grade					
WD	57 (95%)	05 (8.7%)	52 (91.3%)	Reference	>0.05
PD	03 (05%)	01 (33.3%)	02 (66.7%)	5.2 (0.36–67.6)	
Stage, <45 years					
Stage I	34 (56.6%)	03 (8.8%)	31 (91.2%)	Reference	>0.05
Stage II	02 (3.4%)	01 (50%)	01 (50%)	10.3 (0.4–208)	
Stage, ≥45 years					
Stages I and II	15 (21.6%)	01 (6.6%)	14 (93.4%)	Reference	>0.05
Stages III and above	09 (15%)	01 (11.1%)	08 (88.9%)	1.75 (0.08–31.8)	
Lymph node metastasis					
Yes	15 (25%)	02 (13.3%)	13 (86.7%)	Reference	>0.05
No	45 (75%)	04 (8.8%)	41 (91.2%)	0.63 (0.10–3.8)	
Vascular/capsular invasion					
Yes	26 (43%)	03 (11.5%)	23 (88.5%)	Reference	>0.05
No	34 (67%)	03 (8.8%)	31 (91.2%)	0.75 (0.13–4.1)	
BRAF V600E Mutation					
Positive	15 (25%)	02 (13.3%)	13 (86.7%)	Reference	>0.05
Negative	45 (75%)	04 (8.8%)	41 (91.2%)	0.63 (0.4- 1.0)	

TSH = Thyroid-stimulating hormone, WD = well-differentiated thyroid cancer, PD = poorly differentiated thyroid cancer.

Table 8. Association of clinicopathological and clinicoepidemiological characteristic with BRAF protein overexpression.

As an important positive regulator of the MAP kinase signaling pathway, BRAF protein forms a multiprotein complex with MEK (downstream regulatory molecule), hence keeping MAP kinase pathways always on. Therefore, by positive regulation of the MAP kinase signaling pathway, BRAF can accelerate the proliferation of tumor cells. As we could not identify a distinct association between BRAF expression and BRAF mutation in thyroid tumors in accordance with other studies, possibly there might be another potential mechanism of BRAF activation other than mutational events. BRAF copy number gain in thyroid tumors has recently been studied by fluorescence in situ hybridization (FISH) where trisomy, tetrasomy for chromosome 7 was the most common alteration in tumors [108]. Although gains of chromosome 7 lead to the increase in copy number of many genes located on this chromosome, our data suggest that *BRAF* gene may represent an important target for the selection and clonal progression. Furthermore, *BRAF* copy number is directly proportional to amount of BRAF protein [1]. Therefore, it is tempting to speculate that weak stimulation of the MAPK pathway may participate in thyroid carcinogenesis.

7. Analysis of promoter methylation of *TSHR* gene

This study was aimed and designed to analyze the promoter hypermethylation of *TSHR* gene by methylation-specific PCR (MS-PCR) and to correlate it with clinicopathological characteristics of thyroid cancer patients and *BRAF* mutation. For this study, sixty (60) thyroid cancer tissues and their corresponding normal tissues were analyzed. The clinicopathological characteristics of the studied subjects are given in **Table 3**. In case when promoter region was highly methylated (both alleles) only the methylated band was detected and when promoter was partially methylated both methylated and unmethylated bands were detected. The representative picture of promoter hypermethylation of *TSHR* gene by methylation-specific PCR (MSP) is given in **Figure 15**.

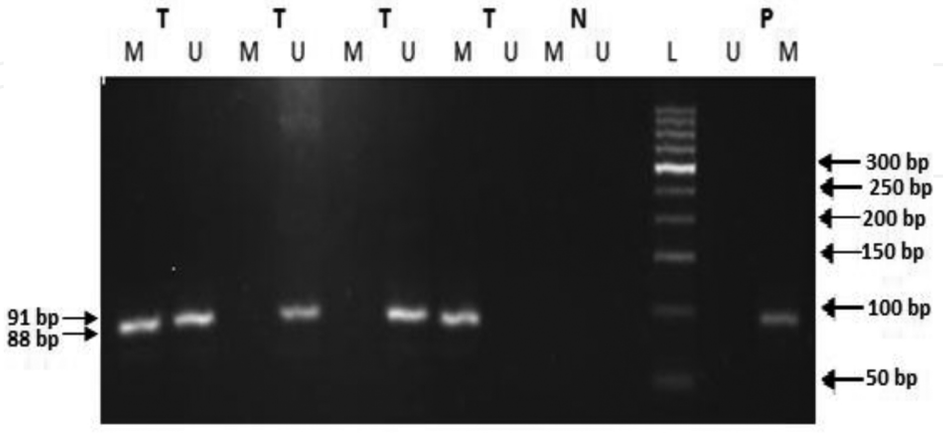

Figure 15. Representative picture of promoter hypermethylation of TSHR gene by MSP (4% agarose). L: 50 bp DNA marker. U (91 bp) indicates presence of unmethylated TSHR. M (88 bp) indicates presence of methylated TSHR. P and N indicate positive and negative controls, respectively. Distilled water was used as negative control in place of DNA.

Variable	Cases n = 60 (%)	*TSHR* methylation (n = 60)		OR (95% CI)	P-Value
		Positive n = 15 (25%)	Negative n = 45 (75%)		
Gender					
Female	48 (80)	12 (25)	36 (75)	Reference	>0.05
Male	12 (20)	03 (25)	09 (75)	1 (0.23–4.3)	
Age					
<45	36 (60)	10 (27.7)	26 (72.3)	Reference	>0.05
≥45	24 (40)	05 (20.8)	19 (79.2)	1.5 (0.45–5.1)	
Dwelling					
Rural	51 (85)	13 (25.5)	38 (74.5)	Reference	>0.05
Urban	09 (15)	2 (22.2)	07 (77.8)	1.2 (0.21–6.5)	
Smoking status					
Nonsmoker	54 (90)	13 (24)	41 (76)	Reference	>0.05
Smoker	06 (10)	02 (33.3)	04 (66.7)	0.63 (0.1–3.8)	
Benign thyroid disease					
Yes	48 (80)	13 (27)	35 (73)	Reference	>0.05
No	12 (20)	02 (16.6)	09 (83.4)	1.7 (0.32–8.8)	
TSH levels					
Elevated	25 (41.6)	10 (40)	15(60)	Reference	**<0.05**
Normal	35 (58.4)	5 (14.2)	30(85.8)	4 (1.1–13.8)	
Histological types					
Papillary	42 (70)	12 (28.5)	30 (71.5)	Reference	>0.05
Follicular	08 (13.4)	02 (25)	06 (75)	1.2 (0.2–6.8)	
Others	10 (16.6)	01 (10)	09 (90)	3.6 (0.4–31.3)	
Grade					
WD	57 (95)	14 (24.5)	43 (75.5)	Reference	>0.05
PD	03 (05)	01 (33.3)	02 (66.7)	0.65 (0.05–7.6)	
Stage, <45 years					
Stage I	34 (56.6)	09 (26.4)	25 (73.6)	Reference	>0.05
Stage II	02 (3.4)	01 (50)	01 (50)	0.36 (0.01–6.3)	
Stage, ≥ 45 years					
Stages I and II	15 (25)	04 (26.6)	11 (73.4)	Reference	>0.05
Stages III and above	09 (15)	01 (11.1)	08 (88.9)	2.9 (0.26–31)	
Lymph node metastasis					
Yes	15 (25)	06 (40)	09 (60)	Reference	>0.05
No	45 (75)	09 (20)	36 (80)	2.7 (0.75–9.4)	
Vascular/capsular invasion					
Yes	26 (43)	06 (23)	20 (77)	Reference	>0.05
No	34 (67)	09 (26.4)	25 (73.6)	0.83 (0.25–2.6)	

TSH = thyroid-stimulating hormone, WD, PD = well and poorly differentiated thyroid cancer.

Table 9. Association of TSHR promoter methylation with different variables of thyroid cancer patients.

The promoter region of *TSHR* gene was found to be methylated in 25% (15 of 60) of the thyroid cancer patients studied. The promoter methylation was found to be 27.7% (10 of 36) in patients <45 years of age compared to 20.8% (5 of 24) in patients ≥45 years of age. When methylation was compared with smoking status of patients, 33.3% (2 of 6) of smokers had methylated promoter region than 24% (13 of 54) of nonsmokers but the association was statistically insignificant ($P > 0.05$). When patients were grouped according to histological types, 28.5% (12 of 42) of PTC patients and 25% (2 of 8) of FTC patients had methylated promoter region, also 10% (01 of 10) of patients having other types of thyroid cancers were having methylation in promoter region ($P > 0.05$). Patients having elevated TSH levels showed strong association with methylation (OR = 4.0, P = 0.02) than patients having normal TSH levels. Association of *TSHR* promoter methylation with other clinicopathological characteristics is given in **Table 9** [126].

TSHR stimulates thyroid epithelial cell proliferation and several key steps in thyrocyte concentration of iodine, including uptake by *NIS* and oxidation before incorporation into Tg by thyroid peroxidase. Excesses or defaults in *TSHR* activity may play a role in thyroid disease and cancer. Aberrant methylation of the *TSHR* gene leads to loss of *TSHR* gene expression [96]. Promoter hypermethylation resulting in decreased expression of *TSHR* and *NIS* may result in a decreased ability to concentrate iodine, rendering ablative doses of 131I ineffective [97]. To summarize, our results showed a higher frequency of *TSHR* gene methylation in thyroid tumors and demonstrated it as a molecular pathway underlying the silencing of this gene. Moreover, the ability to achieve restoration of gene expression by nonnucleoside demethylating agents (such as procainamide) and nucleoside-analogue demethylating agents (such as azacitidine and decitabine) [96] suggests that DNA demethylating agents could be used to improve the efficiency of TSH promoted radioiodine therapy in epithelial thyroid cancers, particularly in those that have lost the response to TSH manipulation.

7.1. Association of TSHR promoter methylation with BRAF mutation spectrum

Now that we found *BRAF* and *TSHR* gene hypermethylation are highly implicated in thyroid tumors, we explored their association in the same group of patients. For this part of study, 60 thyroid cancer tissues and their corresponding normal tissues were analyzed. These were the same patients, wherein mutational analysis of *BRAF* gene and hyper methylation of *TSHR* was carried out. Here, we compared the *BRAF* mutations with *TSHR* promoter methylation. Out of 60 patients, *TSHR* methylation was found in 25% (15 of 60) patients and *BRAF* was found in 25% (15 of 60) patients. Out of 15 patients wherein mutations of *BRAF* gene were found, *TSHR* promoter was methylated in 73.3% (11 of 15) patients (**Table 10**). The presence of methylation in *TSHR* gene was found to be significantly associated with the *BRAF* mutation positive status ($P < 0.05$). Similarly, out of 45 patients, wherein mutations of *BRAF* gene were absent, *TSHR* promoter was methylated in only 8.8% (4 of 45) patients and rest of 91.2% (41 of 45) patients showed absence of *TSHR* promoter methylation (**Table 10**). Among the thyroid cancer patients studied, *TSHR* promoter methylation was significantly greater in patients with *BRAF* mutated (73.3%) than those with wild-type *BRAF* (8.8%) [126].

BRAF mutation	No. of cases (n = 60)	*TSHR* methylation (n = 60)		P-Value
		Positive n = 15 (25%)	Negative n = 45 (75%)	
Present	15	11(73.3%)	04(26.7%)	**0.005**
Absent	45	04(8.8%)	41(91.2%)	

Table 10. Association of BRAF mutation with TSHR promoter methylation.

Several recent *in vitro* and *in vivo* mouse studies have demonstrated that *BRAF* inhibition with small-molecule MAPK pathway inhibitors restores the expression of iodine-metabolizing proteins and increases susceptibility to radioactive iodine (RAI) [99, 127]. Our finding is an addition to the link of promoter methylation of *TSHR* gene with V600E *BRAF* and also represents an interesting further step from previous studies showing promoter methylation as a mechanism in silencing of this gene in thyroid cancer [96]. Our results also conclude that *TSHR* methylation is significantly associated with *BRAF* mutation spectrum. These diagnostic and therapeutic implications of *TSHR* gene methylation and its link with *BRAF* mutation in thyroid tumor clearly deserve further clinical investigation in other ethnic populations as well as our population of Kashmir and because of very few studies done, our results need to be further verified in larger cohort of patients to confirm the link between *BRAF* mutations and *TSHR* promoter methylation.

8. Conclusion

In conclusion, thyroid tumors represent an appropriate model for the study of epithelial neoplastic transformation. Thyroid cancers accumulate a number of alterations at the genomic level, and it has been proposed that genomic instability has a crucial role in the progression of thyroid neoplasms. Recent advances have improved our understanding of its pathogenesis; these include the identification of genetic alterations in *RET, RAS*, and *BRAF* that activate a common effector pathway involving the MAP kinase signaling cascade. Several thyroid-specific protein molecules play a key role in iodide-metabolizing process, including thyroid-stimulating hormone receptor (*TSHR*), sodium iodide symporter (*NIS*), *Tg, TPO*, and the thyroid gene transcription factors TTF-1 and Pax-8. Loss of expression of the genes for these molecules is common in aggressive thyroid cancer and is a sufficient cause for the loss of radioiodine avidity and failure of radioiodine therapy in this cancer.

Although TC is one of the least deadly forms of cancer, research in the field has remained on the cutting edge of science and technology, but better diagnostic tests and predictors of tumor aggressiveness are necessary. Nowadays, novel treatments are being designed based on our enhanced understanding of this disease process. The use of sophisticated genetic tools is generating a wealth of information for the better management of patients with TC.

Our study shows that the *BRAF* mutations as well its protein overexpression characterize the aggressive pathway of thyroid tumorigenesis. The high implication of this gene can thus be exploited for diagnosis and follow-up of thyroid cancer patients. On the other hand, *RAS* genes do not seem to be involved in the thyroid carcinogenesis in our series of patients with thyroid tumors with an exception of a germ line alteration in *HRAS* T81C SNP that moderately increase thyroid cancer risk. Moreover, the ability to achieve restoration of gene expression of thyroid iodide metabolizing genes by demethylating agents (such as azacitidine and decitabine) suggests that DNA demethylating agents could be used to improve the efficiency of radioiodine therapy in epithelial thyroid cancers. We found higher frequency of *TSHR* gene methylation in thyroid tumors, an event underlying the silencing of this gene supporting the above hypothesis about the role of *TSHR* hypermethylation in aggressive thyroid tumors. *BRAF* mutation is associated with silencing of various thyroid iodide-metabolizing genes including *TSHR* and loss of radioiodine avidity, and this is supported by our results that conclude with *TSHR* methylation being significantly associated with *BRAF* mutation spectrum. These diagnostic and therapeutic implications of *TSHR* gene methylation and its link with *BRAF* mutation in thyroid tumor clearly deserve further clinical investigation in other ethnic populations as well as our population of Kashmir.

Because of very few studies done on thyroid cancer from this region and relatively lesser sample size of our study the results need to be further verified in larger cohort of patients to confirm the link between various molecular assaults and thyroid carcinogenesis.

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