Genotypic and Biochemical Investigations in Bangladeshi Patients with Papillary Thyroid Carcinoma

Ph.D. THESIS

A Dissertation Submitted to the University of Dhaka in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy (Ph.D.) in Biochemistry and Molecular Biology

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DECLARATION

I, **Md. Ariful Islam,** hereby declare that the thesis entitled:

"Genotypic and Biochemical Investigations in Bangladeshi Patients with Papillary Thyroid Carcinoma"

submitted to the Department of Biochemistry and Molecular Biology, University of Dhaka, in partial fulfillment of the requirements for the degree of Doctor of Philosophy (PhD), is an original work carried out by me under the supervision of Associate Professor Dr. Md. Ismail Hosen, Department of Biochemistry and Molecular Biology, University of Dhaka, and Professor Dr. Yearul Kabir, Bangladesh Maritime University, Dhaka.

I further declare that this thesis has not been submitted, either in whole or in part, for any degree, diploma, or other qualification at this or any other university or institution.

All sources of information used in this thesis have been duly acknowledged.

Md. Ariful Islam

Date: 14.09.25

CERTIFICATE

This is to certify that the thesis entitled:

"Genotypic and Biochemical Investigations in Bangladeshi Patients with Papillary Thyroid Carcinoma"

submitted by **Md. Ariful Islam**, of the Department of Biochemistry and Molecular Biology, University of Dhaka, has been carried out under our supervision.

To the best of our knowledge, the research work embodied in this thesis is the candidate's own work and has not been submitted, either in part or in full, for the award of any degree or diploma in this or any other institution.

We hereby recommend that this thesis be accepted in partial fulfillment of the requirements for the degree of **Doctor of Philosophy (PhD)** in Biochemistry and Molecular Biology.

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List of Abbreviations

Abbreviation	Full Form/Description
A260	Absorbance at 260 nm
A280	Absorbance at 280 nm
ABI	Applied Biosystems Inc.
ADVIA	Advanced Diagnostic and Immunoassay Analyzer (Siemens)
AI	Artificial Intelligence
AJCC	American Joint Committee on Cancer
AKT	Protein Kinase B (also known as AKT)
ALK	Anaplastic Lymphoma Kinase
AL	Lysis Buffer for DNA Binding (QIAGEN)
AMP	Association for Molecular Pathology
AMPK	AMP-Activated Protein Kinase
AMPure XP	Agencourt AMPure XP Beads
ANNOVAR	Annotate Variation (genomic annotation tool)
anti-Tg	Anti-Thyroglobulin
APC	Adenomatous Polyposis Coli (tumor suppressor gene)
ASCO	American Society of Clinical Oncology
ASIR	Age-Standardized Incidence Rate
ASMR	Age-Standardized Mortality Rate
ATA	American Thyroid Association
ATC	Anaplastic Thyroid Carcinoma
ATE	DNA Elution Buffer (QIAGEN)
ATL	Lysis Buffer (QIAGEN)
ATP	Adenosine Triphosphate
Atellica IM	Atellica Immunoassay System
AUS/FLUS	Atypia of Undetermined Significance / Follicular Lesion of Undetermined Significance

AW1	First Wash Buffer (QIAGEN)
AW2	Second Wash Buffer (QIAGEN)
BLAST	Basic Local Alignment Search Tool
BCL-2	B-cell Lymphoma 2
BMI	Body Mass Index
BMU	Bangladesh Medical University
BRAF	B-Raf Proto-Oncogene, Serine/Threonine Kinase
BRAFV600E	B-Raf Proto-Oncogene Mutation (Val600Glu)
BRCA1	Breast Cancer Type 1 Susceptibility Protein
BRCA2	Breast Cancer Type 2 Susceptibility Protein
bp	Base Pair
CAF	Cancer-Associated Fibroblast
CAP	College of American Pathologists
cBioPortal	cBioPortal for Cancer Genomics
CEA	Carcinoembryonic Antigen
cfDNA	Cell-Free DNA
chr	Chromosome
CI	Confidence Interval
COSMIC	Catalogue of Somatic Mutations in Cancer
CRP	C-Reactive Protein
CSF1R	Colony Stimulating Factor 1 Receptor
CT	Computed Tomography
CTC	Circulating Tumor Cells
ctDNA	Circulating Tumor DNA
CTNNB1	Catenin Beta 1 (Gene coding β-catenin)
CV	Coefficient of Variation
CYP24A1	Cytochrome P450 Family 24 Subfamily A Member 1
cAMP	Cyclic Adenosine Monophosphate
dbSNP	Database of Single Nucleotide Polymorphisms
ddH_2O	Double-distilled Water
delins	Deletion-Insertion

DHCR7	7-Dehydrocholesterol Reductase
DHGTC	Differentiated High-Grade Thyroid Carcinoma
DIT	Diiodotyrosine
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTC	Differentiated Thyroid Carcinoma
dupG	Duplication of Guanine
EBRT	External Beam Radiation Therapy
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-to-Mesenchymal Transition
ERBB4	Erb-B2 Receptor Tyrosine Kinase 4
ERK	Extracellular Signal-Regulated Kinase
ETE	Extrathyroidal Extension
EXOSAP	Exonuclease I and Shrimp Alkaline Phosphatase
EZH2	Enhancer of Zeste Homolog 2
FDG	Fluorodeoxyglucose
FGFR2	Fibroblast Growth Factor Receptor 2
FGFR2-IIIb	Epithelial Isoform of FGFR2
FGFR2-IIIc	Mesenchymal Isoform of FGFR2
FGFR3	Fibroblast Growth Factor Receptor 3
FISH	Fluorescence In Situ Hybridization
FITC	Fluorescein Isothiocyanate
FLT3	FMS-Like Tyrosine Kinase 3
FOXO3	Forkhead Box O3
Fisher's exact test	Statistical test for association in categorical data
FNAB	Fine-Needle Aspiration Biopsy
FTA	Follicular Thyroid Adenocarcinoma
FTC	Follicular Thyroid Carcinoma

FuPa	Fusion Primer Digestion Reagent (Thermo Fisher)
FVPTC	Follicular Variant of Papillary Thyroid Carcinoma
g	Gram
GC content	Guanine-Cytosine Content
GDV	Genome Data Viewer
Geneious	Geneious Prime Software
GeneMANIA	Gene Multiple Association Network Integration Algorithm
GeneRuler™	DNA Size Marker Ladder (Thermo Scientific)
GenBank	Genetic Sequence Database (NCBI)
GLOBOCAN	Global Cancer Observatory
gnomAD	Genome Aggregation Database
GRCh37/hg19	Human Genome Reference Version 19 / Genome Reference Consortium Human Build 37
GRCh38/hg38	Genome Reference Consortium Human Build 38
GTPase	Guanosine Triphosphatase
GWAS	Genome-Wide Association Studies
h	Hour
HDI	Human Development Index
HE	High Efficiency
HIF-1α	Hypoxia-Inducible Factor 1-alpha
HRAS	Harvey Rat Sarcoma Viral Oncogene Homolog
HT	Hashimoto's Thyroiditis
HPT	Hypothalamic-Pituitary-Thyroid
IARC	International Agency for Research on Cancer
IGV	Integrative Genomics Viewer
IMMULITE	Immunoassay Analyzer (Siemens)
Indel	Insertion/Deletion (small genomic variants)
Ion AmpliSeq™	Ion AmpliSeq™ Targeted Sequencing Technology by Thermo Fisher Scientific
ISP	Ion Sphere Particle

JAK	Janus Kinase
KDR	Kinase Insert Domain Receptor (also known as VEGFR-2)
Ki-67	Marker of Cellular Proliferation (Protein Ki-67)
KIT	KIT Proto-Oncogene, Receptor Tyrosine Kinase
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
kb	Kilobase
LM	Local Mode (in Siemens systems context)
LncPVT1	Long Non-Coding Plasmacytoma Variant Translocation 1
LNM	Lymph Node Metastasis
LoD	Limit of Detection
LT4	Levothyroxine
MAPK	Mitogen-Activated Protein Kinase
MEN2	Multiple Endocrine Neoplasia Type 2
MET	MET Proto-Oncogene, Receptor Tyrosine Kinase
mg/dL	Milligram per Deciliter
miRNA	MicroRNA
MiSeq	Illumina MiSeq Sequencing Platform
MIT	Monoiodotyrosine
MHC	Major Histocompatibility Complex
MNG	Multinodular Goiter
MMPs	Matrix Metalloproteinases
MRD	Minimal Residual Disease
MRI	Magnetic Resonance Imaging
mTOR	Mammalian Target of Rapamycin
MTC	Medullary Thyroid Carcinoma
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of Activated B cells
NF water	Nuclease-Free Water
NGS	Next-Generation Sequencing

NIFTP	Noninvasive Follicular Thyroid Neoplasm with Papillary-like Nuclear Features
NIS	Sodium-Iodide Symporter
NLR	Neutrophil-to-Lymphocyte Ratio
NRAS	Neuroblastoma RAS Viral Oncogene Homolog
OR	Odds Ratio
PAC	Paclitaxel (contextual reference)
PAX8/PPARγ	Paired Box Gene 8 / Peroxisome Proliferator-Activated Receptor Gamma Fusion
PCR	Polymerase Chain Reaction
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Death-Ligand 1
PDGFRA	Platelet-Derived Growth Factor Receptor Alpha
PDTC	Poorly Differentiated Thyroid Carcinoma
PET	Positron Emission Tomography
pg/mL	Picogram per Milliliter
PI3K	Phosphoinositide 3-Kinase
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PLR	Platelet-to-Lymphocyte Ratio
PolyPhen-2	Polymorphism Phenotyping v2
PPTC	Papillary Thyroid Carcinoma (used as sample ID prefix)
PTEN	Phosphatase and Tensin Homolog
PTH	Parathyroid Hormone/ Parathormone
PTC	Papillary Thyroid Carcinoma
RAI	Radioactive Iodine
RAS	Rat Sarcoma Viral Oncogene Homolog
RASSF1A	Ras Association Domain Family Member 1A
RB1	Retinoblastoma 1
RefSeq	Reference Sequence Database
RET	Rearranged during Transfection

RET/PTC	RET Proto-Oncogene Fusion with PTC Locus
RLUs	Relative Light Units
RNA	Ribonucleic Acid
ROC	Receiver Operating Characteristic
ROS	Reactive Oxygen Species
RPM	Revolutions Per Minute
RTK	Receptor Tyrosine Kinase
Sanger	Sanger Sequencing Method
SASP	Senescence-Associated Secretory Phenotype
SBS	Sequencing by Synthesis
SEER	Surveillance, Epidemiology, and End Results Program
SEM	Standard Error of the Mean
SIFT	Sorting Intolerant From Tolerant
SLC5A8	Solute Carrier Family 5 Member 8
SMAD4	Mothers Against Decapentaplegic Homolog 4
SMARCB1	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin Subfamily B Member 1
SMO	Smoothened, Frizzled Class Receptor
SN	Solitary Nodule
SNP	Single Nucleotide Polymorphism
SNV	Single-Nucleotide Variant
SPECT	Single-Photon Emission Computed Tomography
STAT	Signal Transducer and Activator of Transcription
STK11	Serine/Threonine Kinase 11
SWI/SNF	Switch/Sucrose Non-Fermentable Chromatin Remodeling Complex
TAE	Tris-Acetate-EDTA
TAM	Tumor-Associated Macrophage
TCGA	The Cancer Genome Atlas Program
TERT	Telomerase Reverse Transcriptase
Tg	Thyroglobulin

TgAb	Anti-Thyroglobulin Antibodies
TGF-β	Transforming Growth Factor Beta
TGFβ1	Transforming Growth Factor Beta 1
TI-RADS	Thyroid Imaging Reporting and Data System
TIMP3	Tissue Inhibitor of Metalloproteinases 3
TKI	Tyrosine Kinase Inhibitors
TME	Tumor Microenvironment
TNF-α	Tumor Necrosis Factor Alpha
TNM	Tumor, Node, Metastasis (Staging system)
TP53	Tumor Protein p53
Tregs	Regulatory T Cells
TR	Thyroid Hormone Receptor
TRH	Thyrotropin-Releasing Hormone
TSH	Thyroid-Stimulating Hormone
TSH3UL	TSH 3-Ultra
TSHR	Thyroid-Stimulating Hormone Receptor
TSP1	Thrombospondin-1
TVC	Torrent Variant Caller
UCSC	University of California, Santa Cruz
uPA	Urokinase-type Plasminogen Activator
uPAR	Urokinase Plasminogen Activator Receptor
USG	Ultrasonography (implied from high-resolution ultrasound)
UTR	Untranslated Region
VAF	Variant Allele Frequency
VDR	Vitamin D Receptor
VEGFA	Vascular Endothelial Growth Factor A
VEGF	Vascular Endothelial Growth Factor
VEP	Variant Effect Predictor
WHO	World Health Organization
Wnt	Wingless-related Integration Site Signaling Pathway

Wnt/β-catenin	Wingless/Integrated and Beta-Catenin Signaling Pathway
X	Fold Coverage (in sequencing depth, e.g., 3000X)
25(OH)D	25-Hydroxyvitamin D
µIU/mL	Micro-International Units per Milliliter
µL	Microliter
°C	Degrees Celsius
χ^2 (Chi-square)	Chi-Square Test Statistic

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Abstract

Background

Papillary thyroid cancer (PTC) is the most frequent type of thyroid carcinoma, and exhibits significant molecular and biochemical heterogeneity both within and between populations. It is indolent in nature, but both its diagnosis and prognosis are difficult to predict given its multiple oncogenic pathways and hormonal derangements. This study explores novel somatic variants and their biochemical correlates in a Bangladeshi PTC cohort, aiming to improve risk assessment and promote personalized cancer treatment in underrepresented populations.

Methods

A cross-sectional comparative study was conducted involving 110 patients with PTC, 100 individuals with benign thyroid conditions, and 100 healthy controls. Targeted next-generation sequencing (NGS) and Sanger validation were employed to identify hotspot and novel intronic variants in NRAS, RB1, HRAS, and RET genes. Concurrently, biochemical profiling included serum thyroid-stimulating hormone (TSH), parathyroid hormone (PTH), calcium, vitamin D, and thyroglobulin (Tg), with correlations analyzed against genotypic status and clinical features.

Results

Three novel variants -NRAS g.7775T>A, NRAS g.7797G>A, and RB1 c.2039T>A (p.Ile680Asn)—were identified, showing significant enrichment among mutation-positive PTC patients ($p < 0.0001$), particularly in non-smoking, non-familial cases, suggesting a sporadic mutational origin. The NRAS g.7797G>A variant was associated with early-stage tumors (T1). In contrast, the RB1 c.2039T>A variant was observed in both early and advanced stages, supporting their involvement in early tumorigenesis.

Biochemically, PTC patients exhibited markedly elevated TSH levels and reduced serum calcium ($p = 0.0305$) compared to benign and healthy controls. Individuals carrying the mutation demonstrated more pronounced hypocalcemia and a significant positive correlation between calcium and vitamin D ($r = 0.3955$, $p = 0.0455$), a pattern not observed in wild-type carriers. No

significant associations were found for Tg or PTH, possibly reflecting heterogeneous expression or unmeasured confounders, such as anti-Tg antibodies. These findings suggest genotype-specific disruptions in calcium–vitamin D–TSH homeostasis and highlight a potential endocrine–genetic crosstalk in PTC pathophysiology.

Conclusion

The current study provides the first integrative molecular–biochemical profile of Bangladeshi PTC patients, identifying novel NRAS and RB1 variants with potential as early detection and intermediate-risk biomarkers. The observed endocrine dysregulation in mutation carriers underscores the interplay between genetic alterations and systemic metabolism. These findings highlight the clinical utility of combining genotypic screening with metabolic markers for cost-effective, precision-guided management of PTC, especially in resource-limited settings. Future studies should focus on functional validation of these variants and the development of region-specific diagnostic and prognostic frameworks.

Keywords:

Papillary thyroid carcinoma, Next-Generation Sequencing, Sanger sequencing, NRAS, RB1, Somatic mutations, TSH, calcium, vitamin D, Biochemical profiling, Precision oncology, Bangladesh, Biomarkers.

Chapter 1

Introduction

1.1 Cancer

Cancer comprises a diverse, biologically complex group of diseases characterized by dysregulated proliferation and survival of genetically altered cells. Under physiological conditions, cellular homeostasis is maintained by tightly orchestrated mechanisms that govern proliferation, differentiation, and apoptosis. In neoplastic transformation, these regulatory networks become compromised, enabling cells to evade programmed cell death, sustain uncontrolled mitotic activity, and acquire invasive capabilities. Malignant cells may subsequently disseminate via hematogenous or lymphatic routes, establishing metastatic foci in distant organs and contributing to systemic physiological disruption (Li et al., 2025).

From a global health perspective, cancer remains a principal contributor to morbidity and mortality. According to GLOBOCAN estimates, approximately 19.9 million new cancer cases and 10 million cancer-related deaths occurred in 2020 (GLOBOCAN, 2022) (Figure 1.1). The geographic distribution of cancer incidence exhibits considerable heterogeneity, influenced by a complex interplay of genetic predisposition, behavioral factors, and environmental exposures (Zhao et al., 2023). Epidemiological projections forecast a substantial escalation in the global cancer burden, with annual incidence expected to reach 28.4 million by 2040 and potentially exceed 35 million by 2050. This trajectory is primarily driven by demographic shifts, population aging, urbanization, and increased exposure to carcinogenic risk factors associated with socioeconomic development and globalization (Singh et al., 2024; Sung et al., 2021).

The most commonly diagnosed malignancies worldwide include breast, lung, colorectal, prostate, and gastric cancers. Among these, lung cancer continues to represent the leading cause of cancer-related mortality, underscoring its aggressive clinical course and limited therapeutic responsiveness in advanced stages (Sung et al., 2021).

A variety of genetic and epigenetic perturbations occur during cancer development, disrupting normal cellular functions. Mechanistic roles include activation of oncogenes, inactivation of tumor suppressor genes, and malfunction in DNA repair pathways (Dakal et al., 2024). These alterations result in the hallmarks of cancer, as identified by Hanahan and Weinberg (2011), namely sustained proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative

immortality, inducing angiogenesis, and activating invasion and metastasis. These classic characteristics support the combative and flexible nature of cancerous cells.

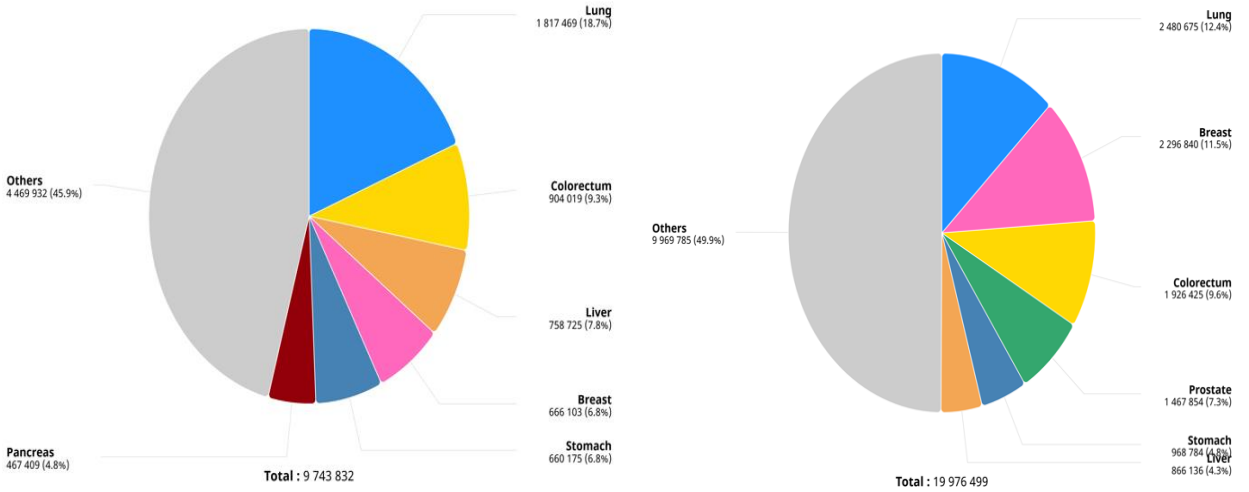


Figure 1.1: Global Cancer Incidence and Mortality in Absolute Numbers for Both Sexes, 2022. (Global Cancer Observatory, 2022)

In recent years, the role of molecular biomarkers in cancer diagnosis, prognosis, and therapy has been highlighted. Biomarkers (e.g., genetic mutations, protein expression profiles, and epigenetic changes) play a crucial role in understanding the molecular pathogenesis underlying cancer and are essential for the implementation of precision medicine. For example, the discovery of specific genetic mutations (1)5, such as mutations in the BRAF or EGFR genes, has led to the development of targeted therapies and improved treatments and outcomes for patients (Jin et al. 2023).

Despite the remarkable advancements, cancer research and treatment continue to face numerous challenges. It is difficult to devise treatment modalities that are universally applicable because tumor heterogeneity, both intra- and inter-patient, impedes such approaches. In addition, the differences in cancer care and outcomes continue to be profound, notably in low- and middle-income countries, where the availability of cancer prevention, early detection, and treatment remains limited (Zahedi et al., 2024; Bizuayehu et al., 2024). Such inequalities further highlight the requirements for worldwide initiatives in enhancing cancer management and producing more equitable and effective care of cancer patients and confronting the escalating global cancer burden.

Cancer remains an intractable enemy that threatens global health. It affects not only individuals but also families and societies, resulting in high emotional, financial, and social costs. Ongoing studies on the molecular mechanisms of cancer and advances in prevention, early detection, and treatment offer optimism that the global burden and patient outcomes will be further improved in the years ahead.

1.2 Hallmarks of Cancer

The "Hallmarks of Cancer" model, first introduced by Hanahan and Weinberg in 2000 and updated in 2011 and 2022, provides a broad framework for examining the fundamental biological processes by which cancer is initiated and progresses (Figure 1.2). It describes specific functionalities that cancer cells develop as they progress from normal cells to tumors. This conceptual framework has revolutionized our perception of tumour biology and remains a cornerstone of cancer research and therapy development (Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011; Hanahan, 2022).

1.2.1 Original Hallmarks of Cancer (2000)

1. **Sustaining Proliferative Signaling:**
   Cancer cells gain the ability to promote unregulated cell proliferation either by generating their own growth signals or by hijacking external signaling pathways. It is commonly driven by mutations in driver oncogenes, such as RAS and EGFR, leading to persistent proliferation (Prior et al., 2020).

2. **Evading Growth Suppressors:**
   Deactivation of tumor suppressor genes, specifically TP53 and RB1, interferes with normal cell-cycle regulation and essential controls on cellular growth, allowing unchecked cellular multiplication (Levine, 2020).

3. **Resisting Cell Death (Apoptosis):**
   Cancer cells acquire molecular characteristics that make them resistant to programmed cell death, often through overexpression of antiapoptotic factors (such as BCL-2) or mutations that impair the function of proapoptotic effector proteins (e.g., TP53) (Elmore, 2007).

4. **Enabling Replicative Immortality:**

   Tumors promote the generation of new blood vessels by increasing angiogenic factors, such as vascular endothelial growth factor (VEGF), to grow in a hypoxic environment and survive (Shay & Wright, 2019).

5. **Inducing Angiogenesis:**

   Tumors promote the generation of new blood vessels by increasing angiogenic factors, such as vascular endothelial growth factor (VEGF), to grow in a hypoxic environment and survive (Carmeliet & Jain, 2000).

6. **Activating Invasion and Metastasis:**

   Epithelial-to-mesenchymal transition (EMT) is a process in which cancer cells gain invasive potential by invading surrounding tissue and traveling to distant organ sites, thereby promoting cancer metastasis (Kalluri & Weinberg, 2009).

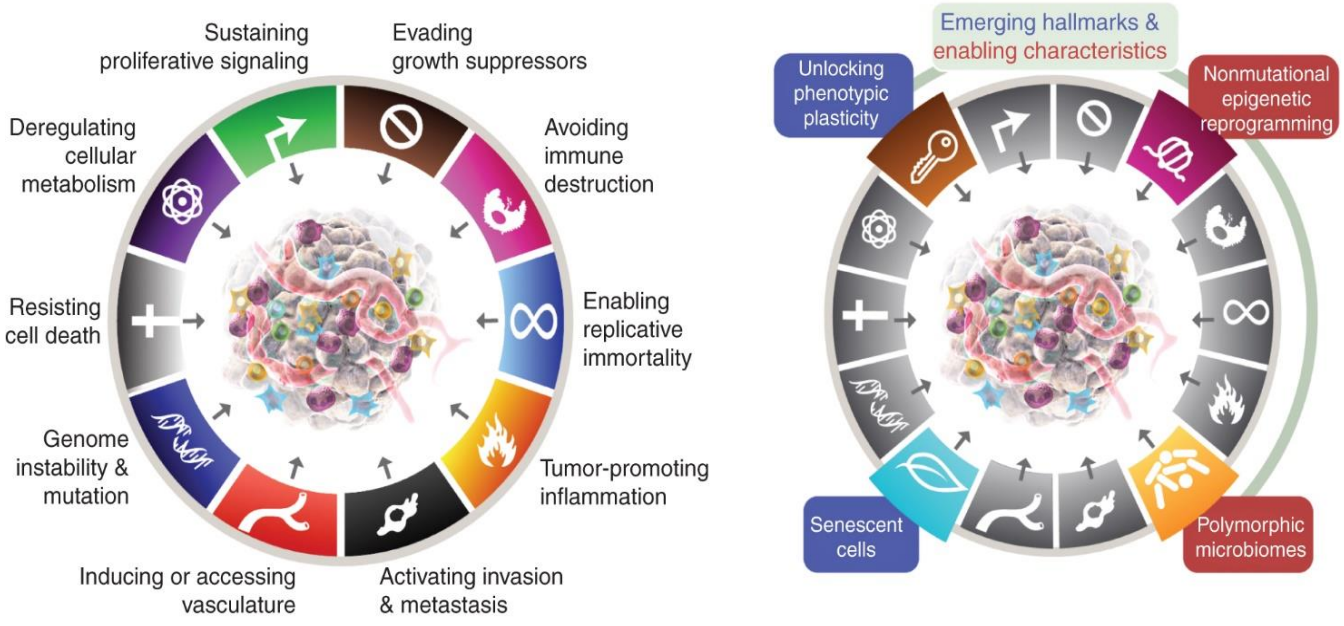


Figure 1.2: The Hallmarks of Cancer: An Overview. (Hanahan, 2022)

1.2.2 Expanded Hallmarks and Enabling Characteristics (2011)

In the 2011 update, Hanahan and Weinberg expanded the original framework to incorporate newly recognized features that further clarify the complexity of cancer development and progression:

1. **Deregulation of Cellular Energetics:**
   Cancer cells change how they metabolize to promote uncontrolled growth, often relying on aerobic glycolysis – even in the presence of oxygen – a process known as the Warburg effect. Such a metabolic shift is designed to provide sufficient energy and biosynthetic elements for the rapid proliferation of the cells (Vander et al., 2009).

2. **Avoiding Immune Destruction:**
   Tumoral cells can develop mechanisms to escape immune surveillance by decreasing major histocompatibility complex (MHC) expression and secreting immunosuppressive factors, such as transforming growth factor-β (TGF-β), that prevent the host from developing an efficient antitumor immune response (Chen & Mellman, 2017).

3. **Genome Instability and Mutation:**
   Genomic instability, which results in high mutation rates, is a defining feature of cancer and among the cues by which the immune system can recognize a tumour. DNA repair defects, including those in the BRCA1 and BRCA2 genes, contribute to this instability and are closely associated with certain types of cancers, such as breast and ovarian cancer (Lord and Ashworth, 2012).

4. **Tumor-Promoting Inflammation:**
   Tumor-promoting functions of chronic inflammation in the tumor microenvironment have been associated with the release of bioactive molecules (e.g., growth factors, survival signals, and pro-angiogenic factors, such as interleukins and TNF-α) that drive tumor growth and metastasis (Grivennikov et al., 2010).

1.2.3 New Dimensions in the Hallmarks Framework (2022)

As a reflection of continuous advances in cancer biology, the Hallmarks of Cancer, in 2022, was extended to include developing notions that encompass the heterogeneity and dynamicity of tumors (Hanahan, 2022):

1. **Unlocking Phenotypic Plasticity:**

   Cancer cells possess the plasticity to change phenotype, allowing them to survive and grow under various microenvironmental conditions and to respond to therapeutic attack. This plasticity contributes to dedifferentiation, stemness, and resistance to therapy.

2. **Nonmutational Epigenetic Reprogramming:**
   In addition to mutations, cancer cells also experience epigenetic alterations that, among other things, affect DNA methylation and histone structure; these changes can affect gene expression and lead to tumor progression (without affecting DNA itself).

3. **Polymorphic Microbiomes:**
   The microbiome composition and variability can influence cancer initiation, progression, and treatment. Microbiomes and their effects on host immune responses and metabolic circuits are of increasing importance in cancer biology (Garrett, 2015).

4. **Senescent Cells:**
   Senescent cells, while incapable of proliferation, are still metabolically active and may secrete a variety of pro-inflammatory and pro-tumorigenic factors. This senescence-associated secretory phenotype (SASP) can reprogram the tumor microenvironment to support tumor promotion.

1.2.4 Implications for Cancer Research and Therapy

The expanding Hallmarks of Cancer concept provides essential clues for developing increasingly targeted and efficient anti-cancer measures:

1. **Targeting Angiogenesis:**
   Anti-angiogenic drugs, such as bevacizumab, target VEGF signaling and block tumor blood vessel formation, thereby limiting nutrient and oxygen delivery to the tumor.

2. **Advancing Immunotherapy:**
   Immunotherapy, a class of anticancer therapy (pembrolizumab) that works with the immune system to stop cancer cells from growing, would make the immune system more effective at identifying and killing tumor cells by blocking a system that turns off immune cells.

3. **Metabolic Intervention:**
   Some therapeutic approaches that target unregulated metabolic pathways used by cancer cells, such as inhibitors of glycolytic enzymes, aim to disrupt the energy production and biosynthetic activities that tumors rely on for continued survival and growth.

The ongoing evolution of the Hallmarks of Cancer epitomizes the progress of cancer scholarship. It emphasises the importance of incorporating emerging biological knowledge into the development of novel therapeutic approaches.

1.3 Thyroid Cancer

Thyroid cancer begins when cells in the thyroid gland change and grow uncontrollably, forming a mass and creating a lump in the neck. These cells can grow together to form a mass or tumor in the thyroid and, in some cases, spread to nearby lymph nodes or other parts of the body (metastasis) (Nguyen et al., 2022).

Thyroid cancer begins in the epithelial cells of the thyroid, a critical endocrine gland situated in the anterior neck. It disrupts the gland's important duties. The thyroid produces essential hormones such as triiodothyronine (T3) and thyroxine (T4), which are involved in metabolism, growth, and energy metabolism. It also produces calcitonin, a hormone involved in calcium regulation. In the event of neoplastic transformation of thyroid cells, these physiological mechanisms could be lost, leading to tumor development (Nikiforov & Nikiforova, 2011).

The clinical course of thyroid cancer is highly variable. Whereas some of these patterns have an indolent course and remain asymptomatic for many years, the others have an aggressive behavior, characterized by early metastatic dissemination and poor clinical results. The worldwide increase in the incidence of thyroid cancer is probably related to better techniques of diagnosis with high-resolution ultrasonography and fine-needle aspiration cytology, thus allowing for the detection of subclinical and early-stage tumors that otherwise would have gone undetected until later in their course (Kitahara & Sosa, 2016).

1.4 Cancer Statistics in Bangladesh

In Bangladesh, cancer has been emerging as one of the alarming public health problems due to high rates of morbidity and mortality. In 2022, there were an estimated 167,256 new cancer cases and 116,598 cancer-related deaths (International Agency for Research on Cancer, 2022). The age-standardized rate of cancer in Bangladesh is 105.6 per 100,000 population and is higher among males (120.8 per 100,000) than that of females (89.5 per 100,000) (Figure 1.3).

Esophageal cancers are the most common cancers among both men and women in Bangladesh (Figure 1.4). Esophageal, lip, and oral cavity, and lung are the top three cancers among males, while breast, cervical, and esophageal cancers are the most frequent in females. These trends are partly explained by lifestyle, including high rates of tobacco and betel quid consumption in the population (International Agency for Research on Cancer, 2022).

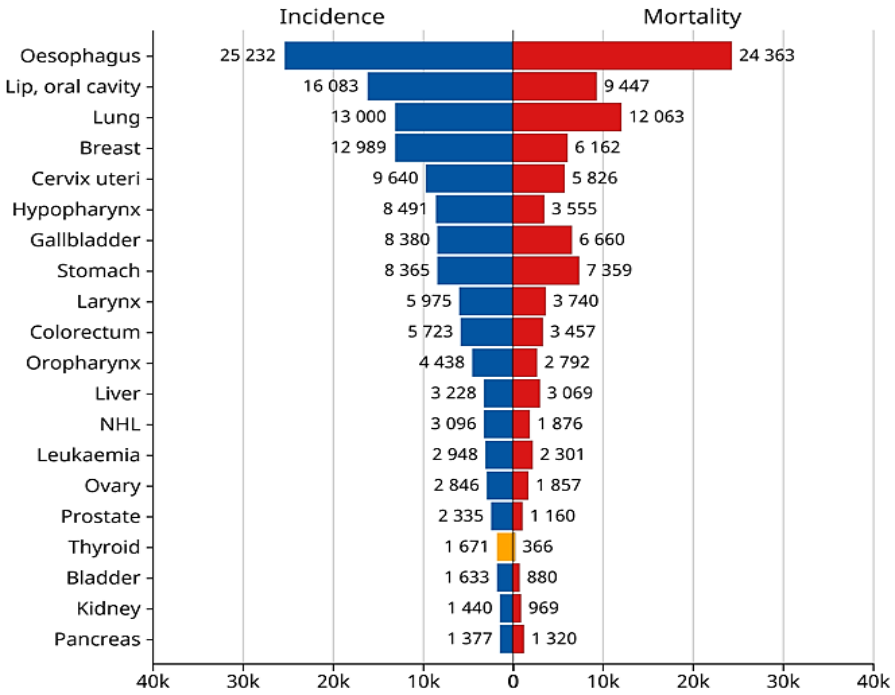


Figure 1.3: Bangladesh 2022: Absolute Numbers of Cancer Incidence and Mortality by Sex. (Global Cancer Observatory, 2022)

	Males	Females	Both sexes
Population	84 788 982	83 096 698	167 885 680
Incidence*			
Number of new cancer cases	94 922	72 334	167 256
Age-standardized incidence rate	120.8	89.5	105.6
Risk of developing cancer before the age of 75 years (cum. risk %)	13.0	9.3	11.3
Top 3 leading cancers (ranked by cases)**	Oesophagus Lip, oral cavity Lung	Breast Cervix uteri Oesophagus	Oesophagus Lip, oral cavity Lung
Mortality*			
Number of cancer deaths	68 591	48 007	116 598
Age-standardized mortality rate	87.8	60.9	74.7
Risk of dying from cancer before the age of 75 years (cum. risk %)	9.7	6.6	8.2
Top 3 leading cancers (ranked by deaths)**	Oesophagus Lung Lip, oral cavity	Oesophagus Breast Cervix uteri	Oesophagus Lung Lip, oral cavity
Prevalence*			
5-year prevalent cases	181 944	164 393	346 337

Figure 1.4: Summary of Cancer Burden in Bangladesh, 2022. (Global Cancer Observatory, 2022)

1.5 Global Trends in Thyroid Cancer

The most common malignancy of the endocrine system is thyroid cancer, which represents over 90% of endocrine neoplasms. However, it is rare, accounting for approximately 2.5% of all cancers globally (Kitahara et al., 2022). While it is one of the least burdened cancers globally, the incidence of thyroid cancer is rising and is the 12th most common cancer diagnosed in the US. Global incidence Approximately 821,214 new cases were recorded worldwide in 2020, making thyroid cancer the 7th most common cancer (Figure 1.5) using GLOBOCAN 2022. It is worth noting that a significant proportion (~91%) of these cases were registered in nations classified as having high or very high Human Development Indicators (HDI) (Shank et al., 2021; GLOBOCAN 2022).

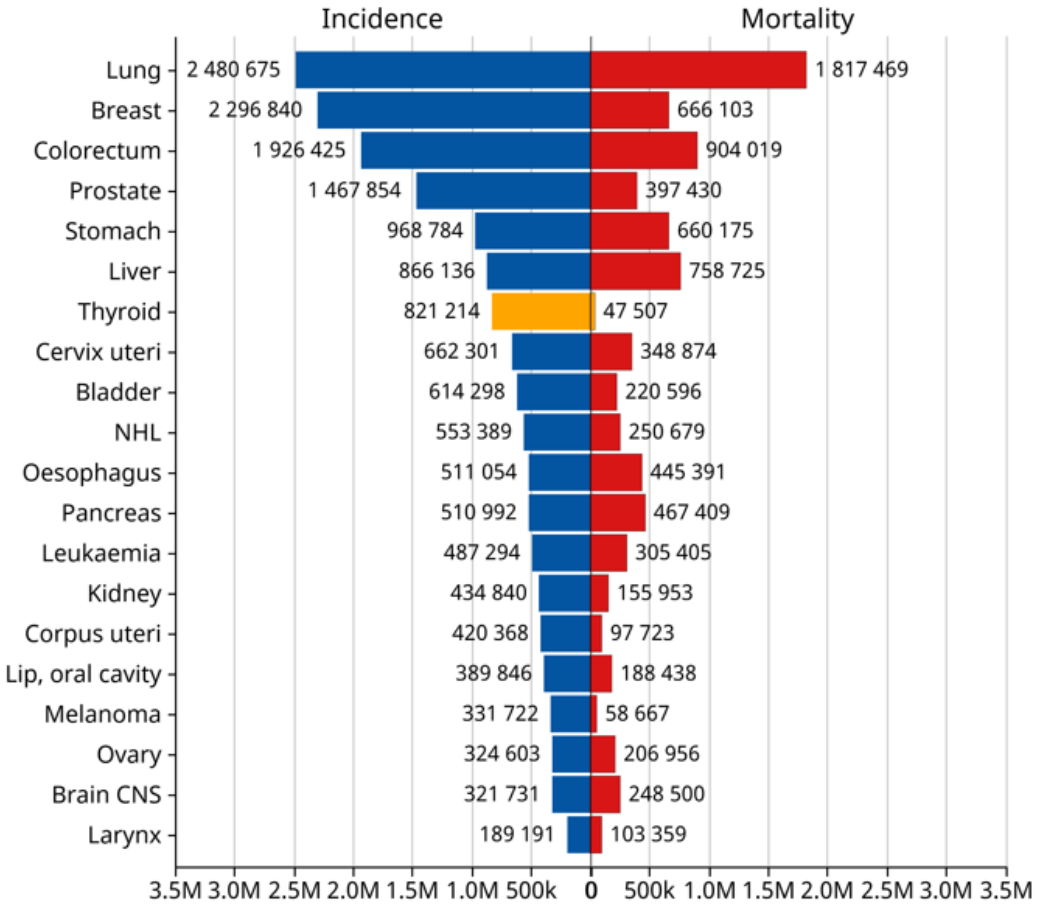


Figure 1.5: Global Age-Standardized Incidence and Mortality Rates of Thyroid Cancer (per 100,000) for Both Sexes, 2022. (Global Cancer Observatory, 2022).

Regarding gender, thyroid cancer has a significant sexual dimorphism at a worldwide level. In 2020, the ASIR was 10.1 and 3.1 per 100,000 in women and men, respectively, indicating a strong female predominance. Although the rates were broadly lower, mortality rates showed a similar pattern, with ASMRs being 0.5 per 100,000 women and 0.3 per 100,000 men (Pizzato et al, 2022) (Figure 1.6). This gender difference, in combination with other anthropometric and environmental risk factors, warrants further scrutiny for the etiology and consequences of thyroid cancer.

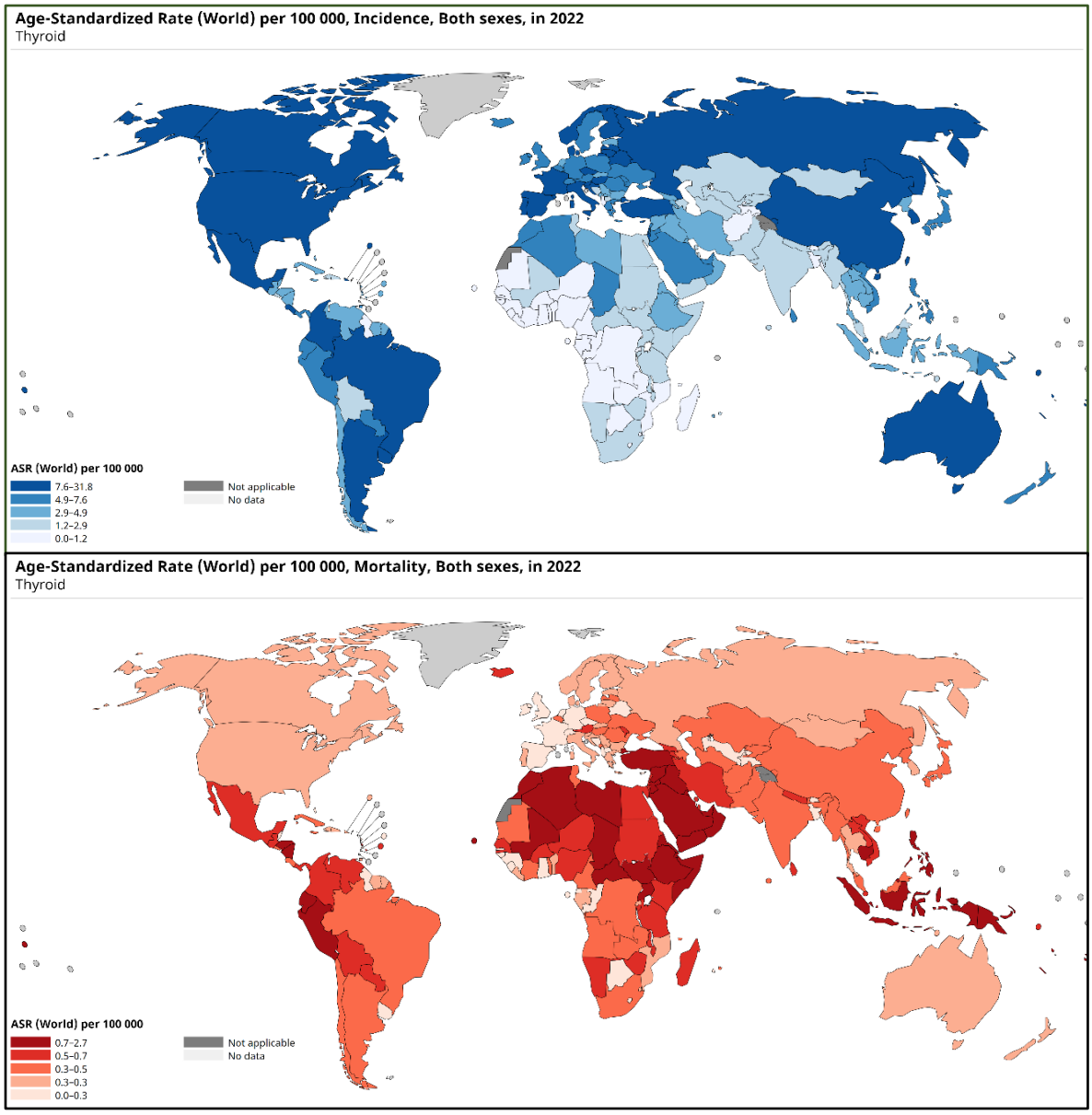


Figure 1.6: Age-Standardized Rates (World) per 100,000 for Thyroid Cancer Incidence and Mortality in Both Sexes, 2022. (Global Cancer Observatory, 2022).

The better prognosis of thyroid cancer is evidenced by the high 5-year relative survival rate, which is around 98.4% in high-income countries (American Cancer Society, 2024). This consensual balance in mortality, despite increasing incidence, attests to the gains from earlier diagnosis and better treatment.

Thyroid cancer was projected to be responsible for about 44,020 new cases and 2,170 deaths in 2024 in the United States. It is much more prevalent in females, at a male/female ratio of approximately 1/3. Of particular importance, the general average age at diagnosis in adult malignancies is younger than 51 years (American Cancer Society, 2024). The growing epidemic of thyroid cancer around the world has, in part, been associated with the extensive application of sophisticated diagnostic imaging modalities, allowing the detection of small, otherwise asymptomatic tumors (Shao et al., 2022). New incidence estimates project an increase from 233,847 in 2019 to 305,587 cases by 2030, a nearly 30% increase (Hu et al., 2024).

1.5.1 Thyroid Cancer in Bangladesh

Thyroid cancer is the 17th most common cancer in Bangladesh, with around 1,671 new cases and 366 deaths per year (GLOBOCAN 2022 – Bangladesh) (Figure 1.2). As elsewhere, the rate of thyroid cancer in the country is also increasing. Cadaveric studies from Bangladesh have suggested that the prevalence of thyroid cancer may be around 2%. Thyroid nodules, in contrast, are discovered in approximately 4-5% of the general population. Notably, malignancy rates also vary between solitary thyroid nodules (about 10%) and multinodular goiters, suggesting the clinical relevance of nodule features for determining thyroid cancer risk (Lim et al., 2017; Matin et al., 2021).

1.6 Types of Thyroid Cancer

Thyroid cancer is a heterogeneous group of cancers arising from different cell types that make up the thyroid gland, with characteristic pathological and clinical profiles. The major subtypes include:

1. **Papillary Thyroid Carcinoma (PTC)**

   As the most prevalent subtype, PTC is responsible for approximately 84% of all thyroid cancer cases (Figure 1.7). It predominantly occurs in adults in their 30s and 40s, with a female predominance. PTC is slow-growing, but most cases metastasize to cervical lymph nodes. Prognosis is generally good when diagnosed early and managed appropriately (Boucai et al., 2024).

2. **Follicular Thyroid Carcinoma (FTC)**

   FTC accounts for approximately 4% of thyroid cancers and arises from follicular epithelial cells. It is generally diagnosed in adults aged 40 to 60. In contrast to PTC, FTC shows a higher tendency toward hematogenous dissemination, with distant organ involvement, e.g., the lung and bone. Hürthle cell carcinoma is classed as a histological variant within this group to some extent (Grønlund et al., 2021).

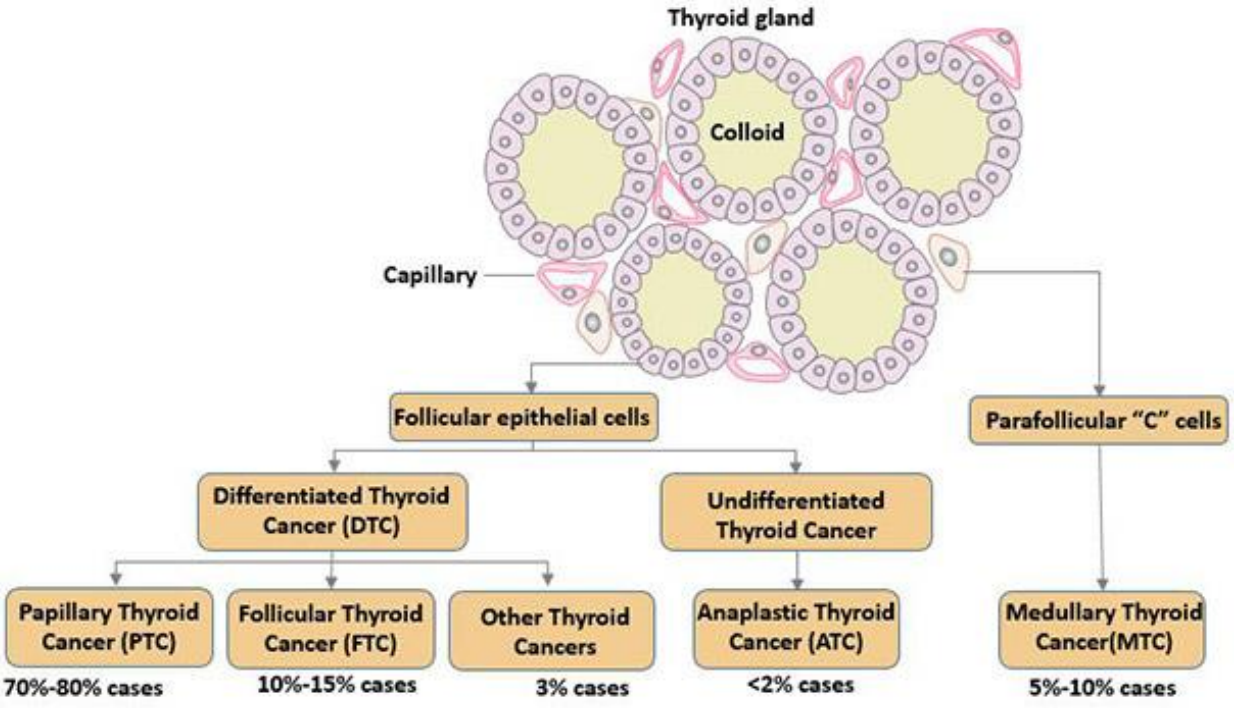


Figure 1.7: Types of Thyroid Cancer. (cusabio.com)

3. **Medullary Thyroid Carcinoma (MTC)**

   Derived from parafollicular C cells, MTC accounts for about 4% of the thyroid cancer burden. These cells secrete calcitonin, a hormone used as a biomarker for diagnosis and monitoring. 25% of MTC cases are familial and are frequently associated with RET proto-oncogene mutations and syndromes (e.g., MEN2). MTC tends to spread to areas including the liver and lungs (Machens et al., 2022).

4. **Anaplastic Thyroid Carcinoma (ATC)**

   ATC is an uncommon but very aggressive malignancy, representing 1% of all thyroid cancers. It is known for rapid growth, insensitivity to standard treatments, and an overall poor prognosis with a median survival of approximately 6.5 months. ATC mainly occurs in older populations and frequently exhibits marked locoregional invasiveness (Jannin et al., 2022).

5. **Poorly Differentiated Thyroid Carcinoma (PDTC)**

   PDTC accounts for about 5% of all thyroid malignancies and lies on a biological and clinical continuum between well-differentiated thyroid carcinomas (WDTCs), including PTC and FTC, and undifferentiated ATC. It is more aggressive than WD types, but is usually less aggressive than ATC (Tong et al., 2022).

6. **Uncommon Thyroid Malignancies**

   - **Thyroid Lymphoma:** Uncommon neoplasm derived from lymphoid tissue of the thyroid gland, which is often seen with autoimmune thyroiditis. Therapy typically involves chemotherapy and radiotherapy, consistent with standard lymphoma regimens (Rovira et al., 2023).
   - **Thyroid Sarcoma:** This is an uncommon malignancy that arises in the mesenchymal tissue of the thyroid. Treatment could be multimodal, including surgery, radiation, and even chemotherapy due to its aggressive nature (Seyed et al., 2021).

1.7 Thyroid Hormone

1.7.1 Thyroid Hormones: T_4 and T_3

Thyroid hormones: thyroxine (T_4) and triiodothyronine (T_3), are vital endocrine controllers of a variety of biological functions, such as metabolism, growth, and tissue differentiation. These hormones have wide-ranging effects on almost all body compartments, including the regulation of basal metabolic rate, protein synthesis, and cellular energy use. They are crucial for neurodevelopment during fetal and early post-infantile life (Salas-Lucia et al., 2022).

1.7.2 Biosynthesis and Secretion

Synthesis of thyroid hormones begins (Figure 1.8) with the transport of iodide ions into the thyroid follicular cells by the sodium-iodide symporter (NIS) at the basolateral membrane. Iodide is then transferred into the cells and into the follicular lumen, where it is oxidized by thyroid peroxidase (TPO). The iodine that has been oxidized is then covalently linked to selected tyrosine side chains of thyroglobulin to give monoiodotyrosine (MIT) and diiodotyrosine (DIT) (Riesco-Eizaguirre et al., 2021).

These hormones are synthesized through the linking of iodinated tyrosines: one molecule of MIT with one molecule of DIT generates T_3, and two DIT molecules connect to form T_4. These iodinated thyroglobulin complexes are extracellularly retained in thyroid follicular colloid until utilization (Herranen et al., 2025).

Following stimulation with TSH, released from the anterior pituitary, thyroglobulin is internalized via endocytosis, processed within lysosomes, and the bioactive hormones T3 and T4 are released into the blood. While T_4 is produced in larger amounts, T_3 is the more biologically active form and is predominantly formed in the periphery, mainly the liver and the kidneys, by deiodination of T_4 (Benvenga et al., 2018).

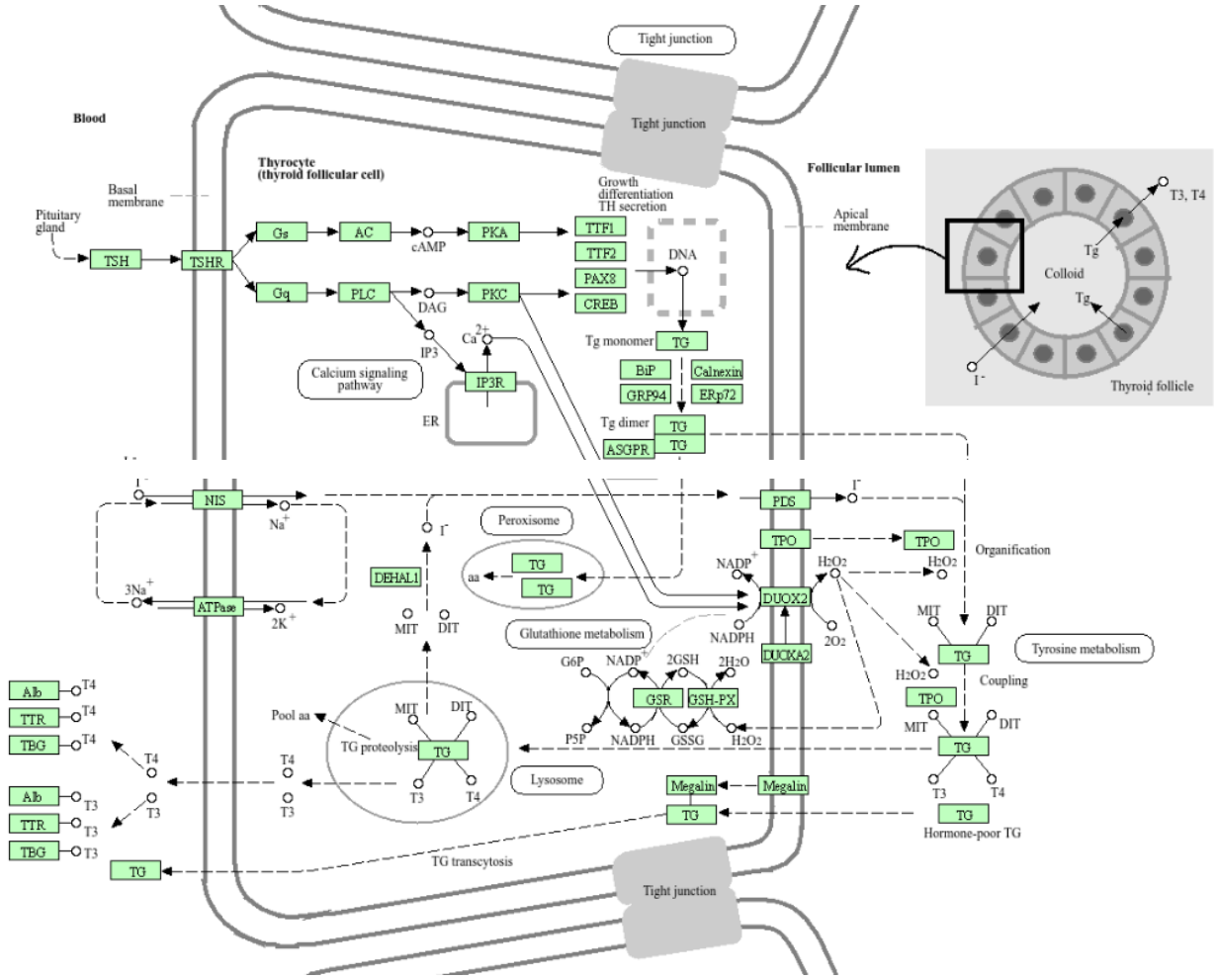


Figure 1.8: Thyroid Hormone Synthesis. (KEGG Pathway)

1.7.3 Regulation of Thyroid Hormone Levels

The hypothalamic-pituitary-thyroid (HPT) axis coordinates the production and release of thyroid hormones, and represents a typical endocrine feedback loop (Figure 1.9). The process starts in the hypothalamus when thyrotropin-releasing hormone (TRH) is released, which causes the anterior pituitary gland to make and release thyroid-stimulating hormone (TSH). This, in turn, stimulates TSH to bind to the receptors on the thyroid follicular cells and to increase production and secretion of thyroxine (T_4) and triiodothyronine (T_3) (Feldt-Rasmussen et al., 2021).

Increasing levels of T_4 and T_3 in the circulation feed back both at the hypothalamus and the anterior pituitary gland to inhibit further release of TRH and TSH. This negative feedback loop helps maintain thyroid hormone levels within a narrow physiological range, thereby controlling metabolic and developmental homeostasis (Al-Suhaimi et al., 2022).

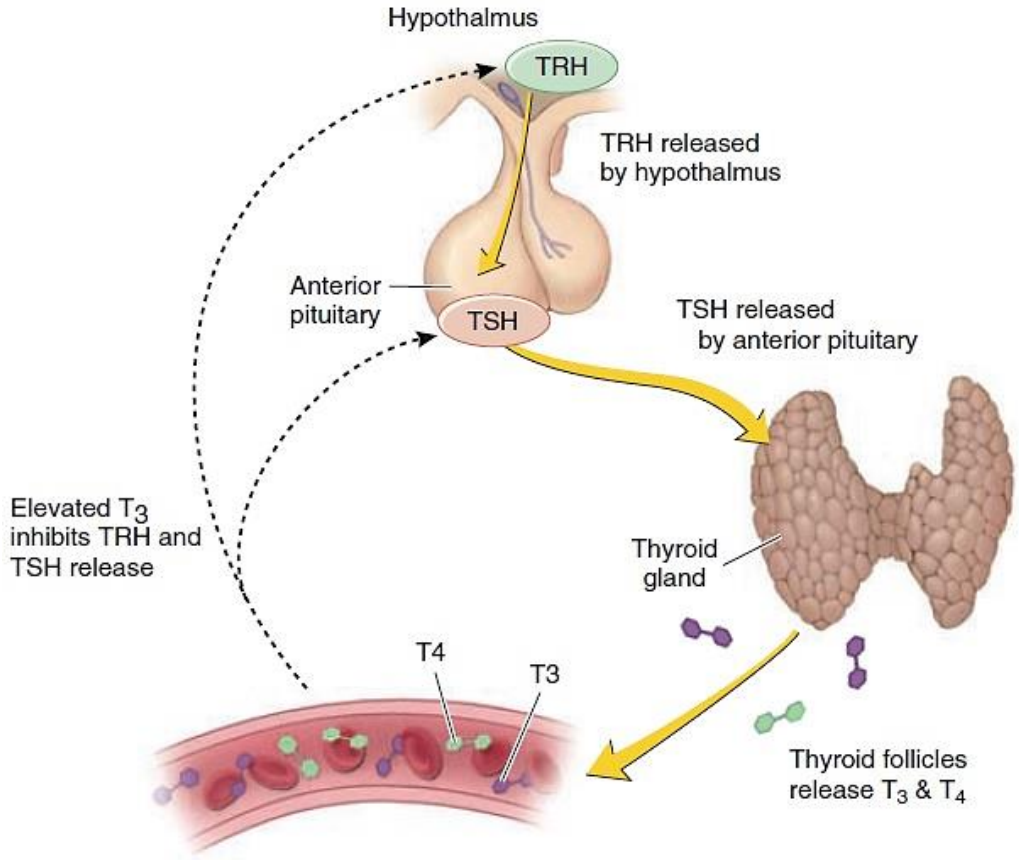


Figure 1.9: Regulatory Mechanisms of Thyroid Hormone Secretion. (BrainKart, 2017)

1.7.4 Mechanism of Action

The physiological effects of thyroid hormones are partly mediated by their binding to nuclear intracellular receptors (TRs) in target organs. After being transported into a cell, physiologically less active hormone thyroxine (T_4) is converted by a (usually intracellular) deiodinase to T_3, which binds with higher affinity to the thyroid hormone receptor and causes a conformational change in the receptor protein. After the conformational change, the receptor complex dissociates from

caveolin (if caveolin is bound) or binds to nuclear export protein division cycle 4 (NEDD4) bound by ubiquitin. Although thyroxine (T_4) is the main form of thyroid hormone within the blood, it acts mainly as a prohormone, being converted to T_3 in peripheral tissues (Brtko, 2021; Tanda et al., 2022).

The effects of thyroid hormone action, such as stimulated mitochondrial function, increased heat production (thermogenesis), and increased protein synthesis, are vital in the body for maintaining some systemic metabolic equilibrium and energy homeostasis (Zekri et al., 2021).

1.7.5 Physiological Roles of Thyroid Hormones

1. **Metabolic Regulation**:

   Thyroid hormones play a pivotal role in modulating basal metabolic rate by activating mitochondrial function, increasing ATP synthesis and oxygen consumption. They participate in the catabolism of fats, proteins, and carbohydrates, which are closely related to energy homeostasis and body weight (Sinha et al., 2024).

2. **Cardiovascular Function**:

   They exert a considerable influence on cardiac function; they increase heart rate, myocardial contractility, and cardiac output. Furthermore, they exert peripheral vasodilatory effects to reduce vascular resistance and enhance organ perfusion according to metabolic needs (Soetedjo et al., 2024).

3. **Neurodevelopment**:

   Thyroid hormones are vital for the maturation of the CNS (central nervous system), especially during the fetal and early postnatal period. Insufficient support during these vulnerable periods can lead to long-lasting neurodevelopmental impairments, resulting in long-term deficits in cognitive function (Vujovic et al., 2025).

4. **Heat Production**:

   They play a role in thermoregulation by stimulating heat production, mainly through the induction of brown adipose tissue thermogenesis, a central mechanism for body temperature regulation (Taylor et al., 2023).

1.7.6 Clinical Significance of Thyroid Hormones

1. **Hypothyroidism**:

   It is a result of the body not producing enough thyroid hormone, and is characterized by symptoms such as feeling sluggish, gaining weight, and intolerance to cold temperatures. Primary causes include autoimmune-mediated thyroid gland destruction (e.g., Hashimoto's thyroiditis) and iodine deficiency. Conventional treatment consists of hormone replacement treatment by synthetic levothyroxine (Wilson et al., 2021).
2. **Hyperthyroidism**:

   Characterised by the persistent production of excessive amounts of thyroid hormones, features including involuntary weight loss, heat intolerance, and a rapid heart rate accompany hyperthyroidism. One of the significant causes of hyperthyroidism is Graves' disease, for which treatment options include antithyroid drugs, radioactive iodine ablation, or surgery (Wiersinga et al., 2023).

1.8 Risk Factors for Thyroid Cancer

Papillary thyroid carcinoma (PTC) may be the result of a complex combination of inherited/genetic factors, environmental components, environmental influences, and individual lifestyle. The precise mechanisms underlying PTC have not been fully elucidated; however, several contributing factors have been identified (Faten et al., 2019).

The epidemiological study of thyroid cancers has also faced problems in diagnosis, particularly in identifying the aetiological factors involved. This challenge is quite clearly attributed to the indolent course of a majority of thyroid tumors, usually discovered incidentally (on imaging tests

performed for other health issues, or when being examined for another benign thyroid condition). Therefore, in thyroid cancer, there is a widespread use of the term “risk factor” to indicate a condition or an exposure for which there is a high probability of developing the disease (Pellegriti et al., 2013).

For a more discriminating approach, causative or etiologic risk factors fulfilled specific critical criteria of causation, such as temporality, consistency, strength of association, dose-response, specificity, plausibility, and coherence with current knowledge. Conversely, modifiable risk factors are variables or behaviors that can be modified to decrease or increase the probability of developing thyroid cancer (Kitahara et al., 2022).

1.8.1 Radiation Exposure

Radiation exposure is a known environmental factor for the risk of induction of thyroid cancer. Depending on the type of exposure (external or internal, accidental or occupational, therapeutic), the radiation levels that reach the thyroid gland may differ, which, in turn, may determine its carcinogenic potential (Saenko et al., 2024).

The known risk factors for papillary thyroid cancer (PTC) include ionizing radiation and are well-established, especially at a young age. The connection between radiation and PTC first became evident based on clinical presentation and was later supported by epidemiological data. A pooled analysis of seven cohort studies demonstrated a linear dose-response, best described by a line representing linear >l (Fig. 57.1, left panel), and the association was strongest in subjects exposed at a young age (Veiga et al., 2016). This age group, including children under five years of age who were exposed during events such as the 1986 Chornobyl nuclear explosion, demonstrated a dramatic increase in cases of PTC. These tumors were frequently more aggressive histologically, showing characteristics such as a higher frequency of solid variant, which is very uncommon in unexposed groups, illustrating the greater sensitivity of young thyroid tissue to radiation (Iglesias et al., 2017; Handkiewicz et al., 2016; Suzuki et al., 2015).

Medical radiation sources of ionizing radiation (i.e., computed tomography and radioactive iodine therapy) also cause increased risk of thyroid cancer. Although CT accounts for only about 15% of imaging procedures, it contributes more than 50% of the overall medical radiation exposure, especially to the thyroid gland during head and neck imaging. Even modestly low total exposures (50–100 mGy) have been shown to double the thyroid cancer risk (Su et al., 2014). In the same line, RAI, used for the treatment of hyperthyroidism or thyroid cancers, has been linked to increasing incidences of thyroid as well as other solid tumors. Exposure to higher RAI doses has also been associated with increased deaths from cancers, including, for example, breast cancer (Shim et al., 2021; Saenko et al., 2024).

1.8.1.1 Molecular Mechanisms of Radiation-Induced PTC

While RET/PTC rearrangement is more frequent in PTC that developed following radiation than in PTC that occurred without radiation, the specific mechanisms underlying radiation-induced PTC remain unclear.

The carcinogenic potential of radiation is mainly due to DNA damage, especially double-strand breaks (DSB), which can lead to somatic mutations and chromosomal changes that contribute to the initiation phase of tumorigenesis (Hecht et al., 2024; Su et al., 2016). RET/PTC rearrangement, which is prevalent among PTC patients associated with the Chornobyl incident Liu et al., 2024, is a central molecular feature of radiation-related PTC.

Elisei et al. (2001) reported RET/PTC rearrangements in both sporadic and radiation-induced PTC, suggesting a common pathway in thyroid carcinogenesis. Additional studies by Hecht et al. emphasize that postirradiation replicative stress in thyroid cells causes the focused formation of double-strand breaks in the RET gene, which serve as a starting point for chromosomal translocations specific to these cancers (Hecht et al., 2024). These results highlight the importance of ionizing radiation in the pathogenesis of RET/PTC-driven thyroid carcinogenesis.

1.8.2 Benign Thyroid Diseases

Benign thyroid disorders such as thyroid nodules, goiter, thyroiditis, hyperthyroidism, and hypothyroidism commonly occur before thyroid cancer is diagnosed (Schonfeld et al., 2020;

Kitahara et al., 2018). But it is unclear if these conditions directly promote or retard malignancy. Diagnosis of thyroid cancer is frequently an incidental finding in the clinical assessment or follow-up of these benign diseases. This detection bias is reduced by excluding thyroid cancer cases that were diagnosed shortly after a benign disease (Macvanin et al., 2023).

Recent epidemiologic studies have investigated the risk of thyroid cancer in relation to benign thyroid diseases. An extensive cohort study found increased risks of benign thyroid nodules and adenomas, with relative risks of approximately 30 for nodules and 5 for adenomas, and of goiter, with a relative risk of 5 (Kitahara et al., 2018). The association between functional thyroid diseases (i.e., hyperthyroidism and hypothyroidism) and risk of cancer, however, is less stable. Although several studies report a positive association, others report no such correlation, indicating no consensus in the available data (Yuan et al., 2020). Similarly, the information concerning autoimmune thyroiditis and its possible role in thyroid carcinogenesis is scant and inconclusive (Resende et al., 2017).

Given the prolonged surveillance and diagnostic imaging frequently used in individuals with benign thyroid conditions, there may be a tendency for coincidental thyroid cancer ascertainment, thereby making it difficult to separate real causal relations from detection bias (Kitahara et al., 2023). Solitary thyroid nodule (SN) has the highest malignancy rate (about 5%) among benign thyroidal diseases, including multinodular goiter [2]. However, findings are highly context-specific, depending on iodine sufficiency and geographical location, as evidenced by recent meta-analytic research (Rehman et al., 2022; Lau et al., 2021; Mu et al., 2022). Future studies are needed to elucidate the effect of genetic predisposition, environmental exposure, and dietary factors on the progression from benign thyroid disease to malignancy (Shen et al., 2024).

1.8.3 Iodine Intake

Iodine is an essential bioelement for the normal regulation of thyroid metabolism, and its balance is crucial during key periods of life, such as the perinatal period and pregnancy, when the thyroid is particularly susceptible to the effects of antithyroid agents, especially iodine excess. Long-term iodine deficiency can result in elevated TSH, and elevated TSH has been linked to an increased risk of FTC, presumably due to continuous TSH stimulation. Nevertheless, their mechanisms of

action have not yet been completely unraveled (Bogović et al., 2020). Iodine status is associated with histological patterns of thyroid cancer based on epidemiological data; as found, FTC tends to account for a larger portion of thyroid cancer cases in iodine-deficient areas, whereas PTC is more common in iodine-sufficient regions (Kwon et al., 2024; Zhang et al., 2022; Fan et al., 2021). For instance, after the Chornobyl nuclear disaster, an increased incidence of thyroid cancer was noted in regions with an inadequate iodine intake, which reflected the capacity of iodine to regulate the risk of cancer (Bogović et al., 2020).

Nevertheless, it remains challenging to establish a direct link between I intake and the risk of TC, also due to methodological limitations of dietary assessments and the paucity of long-term prospective studies (Candido et al., 2023). There is some evidence that iodine supplementation programs have changed the balance from FTC to PTC in former iodine-deficient areas with little concern about the oncogenic capacity of iodine excess exposure (Kim et al., 2021).

Specific dietary sources of iodine and their potential relevance to thyroid carcinogenesis have also been investigated recently. A Japanese cohort study found that high seaweed intake was directly associated with increased PTC risk, although this association has not been replicated broadly (Smyth et al., 2021). By contrast, pooled international analysis has suggested that fish intake may be protective against thyroid cancer in iodine-deficient areas, whereas in iodine-replete areas, an inverse association has been observed between cruciferous vegetable intake and thyroid cancer risk (Hong et al., 2024; Bath et al., 2024).

In addition to modulating cancer rates, iodine is likely to affect tumor biology. Elevated iodine exposure was associated with a higher proportion of BRAF-mutated PTCs, a genetic aberration frequent in these cancers and possibly contributing to the worldwide surge in PTCs (Xiao et al., 2022; Kim et al., 2018). These results indicate that iodine has a more general role in influencing the risk of thyroid cancer and in changing the molecular and histological features of this type of tumor. Furthermore, stronger prospective studies with valid and reliable pre-diagnostic iodine levels are needed to elucidate these relationships (Lin et al., 2023).

1.8.4 Thyroid-Stimulating Hormone (TSH)

TSH is a primary hormonal controller of thyroid physiology, governing thyroid cell proliferation, differentiation, and hormone synthesis. The association between high TSH and the risk of developing PTC is increasingly recognized, revealing new biological mechanisms and prognostic considerations.

1.8.4.1 Biological Role of TSH in Thyroid Carcinogenesis

The action of TSH is mediated by the thyroid-stimulating hormone receptor (TSHR), which is expressed in benign and malignant thyroid tissues. This triggers the Gαs–adenylyl cyclase–protein kinase A–cAMP pathway, which enhances proliferation and hormone production in thyroid cells (Leandro et al., 2023). Chronically elevated TSH may promote neoplastic transformation, especially in oncoprotein mutations such as BRAFV600E and TERT promoter mutations, which are frequently found in PTC (Kim et al., 2022).

1.8.4.2 Clinical Associations between TSH and PTC Risk

Several clinical studies have demonstrated a positive association between serum TSH levels and the occurrence of PTC. The subjects with TSH levels in the upper quartile of the reference range have a much greater risk of PTC and may have more aggressive tumor features (Fan et al., 2024; Liu et al., 2024). Alaraifi et al. (2023) reported that patients with malignant thyroid lesions had higher TSH values than those with benign nodules. Furthermore, prolonged exposure to elevated TSH enhances the likelihood of somatic mutations and cellular proliferation (Liu et al., 2024). Of note, sex-specific patterns have been observed: females with TSH suppression and males with TSH elevation are at greater risk of PTC. An inverse correlation between the risk of PTC and TSH levels within the physiological range was established in both males and females in these mean experimental models (Huang et al., 2017).

1.8.4.3 Molecular Insights: TSH and Genetic Pathways in PTC

Furthermore, experimental studies have shown that TSH can promote oncogenic transformation via a cAMP-dependent pathway, thereby making thyroid cells more susceptible to oncogenic

mutations, such as BRAFV600E (Ran et al., 2022). TSH could also regulate the levels of long non-coding RNAs (lncRNAs), such as LncPVT1, which modulates cancer cell proliferation by regulating TSHR expression through EZH2-mediated epigenetic regulation (Macvanin et al., 2023; Zhao et al., 2021; Peng et al., 2020). Moreover, genome-wide association studies (GWAS) have identified several low-frequency variants (e.g., rs965513, rs944289) associated with lower TSH levels and greater susceptibility to thyroid cancer, indicating a genetic component that may underlie this hormone–cancer connection (Zhang et al., 2020).

1.8.4.4 Diagnostic and Prognostic Relevance of TSH

In addition to the risk estimation, the diagnostic and prognostic relevance of TSH levels in thyroid cancer has been investigated. Elevated TSH levels have been correlated to advanced tumor stage and recurrence at surgery. Receiver operating characteristic (ROC) analysis has defined a TSH level of 2.4 mIU/L as a diagnostic threshold with sensitivity and specificity greater than 70% for differentiating malignant from benign thyroid nodules (Liu et al., 2024). Moreover, elevated TSH enhances the sensitivity of thyroglobulin as a diagnostic marker for PTC relapse (Prpić et al., 2018).

1.8.4.5 Modifying Factors in the TSH–PTC Relationship

Several exogenous factors may affect the correlation between TSH and PTC. Higher body mass index (BMI) and excessive iodine exposure have both been found to correlate negatively with TSH levels (Liu et al., 2024; Kim et al., 2023; Son et al., 2018). Although serum uric acid has been associated with thyroid nodule formation, its direct influence on thyroid carcinogenesis remains unclear (Huang et al., 2022).

To summarize, TSH is a potent mitogen and regulator in thyroid oncogenesis. Serum levels are invariably associated with elevated PTC risk, aggressive tumor behavior, and recurrence. The interactions between TSH and genetic mutations, and their molecular signaling, are complex, underscoring the need for further study. From a clinical perspective, attentive follow-up of TSH levels and judicious use of TSH-suppressive therapy with levothyroxine are key measures in managing both the risk and recurrence of PTC.

1.8.5 Gender

Thyroid cancer has one of the most significant gender disparities of any cancer, with rates 2-5 times higher in women than men. This observance underscores female sex as a major epidemiological risk factor for thyroid cancers, most notably papillary thyroid carcinoma (PTC) (Remer et al., 2022; LeClair et al., 2021). Based on population data (derived from the Surveillance, Epidemiology, and End Results [SEER] program and the International Agency for Research on Cancer [IARC]), the average male-to-female ratio for thyroid cancer is about 0.3. Diagnoses also differ by sex, with earlier age at diagnosis among women (40–49 years, the reproductive years) compared with men (60–69 years) (Kitahara et al., 2022; Bray et al., 2022).

Although thyroid cancer is seen more commonly in females, compared to their female counterparts, men tend to have a more aggressive clinical presentation. Men are more predisposed to be accompanied by larger tumors, more LNM, higher TNM stage, and more mortality person-time (Suteau et al., 2021; Wang et al., 2022). Analyses of tissue specimens have shown that early-stage (T1-T2) and less aggressive (tumor subtypes) are over-represented in women, whereas advanced-stage (T3-T4) and more aggressive (histologic variants) are found much more often in men (Lorenz et al., 2020; Lu et al., 2023).

The following explanations have been suggested to explain these discrepancies in the reported sex-related effects:

1. **Hormonal and Physiologic Influences:** Estrogen and androgen hormones are known contributors to thyroid cell growth and cancer, with potential sex-dependent activity of tumor sex hormone receptors. Differential expression of sex hormone receptors at both the mRNA and protein levels in normal and neoplastic thyroids indicates a biological substrate for sex-specific tumor behavior (Suteau et al., 2021; Zhang et al., 2023).
2. **Presentation and prognosis:** Men are found at an older age than women, and suffer from shorter disease-free survival. Investigations, such as those conducted by the SEER and Canadian registries, indicate that male gender is a risk factor for increased recurrence and a more unfavorable prognosis (Li et al., 2021; Sawka et al., 2021; Zahedi et al., 2020).

3. **Sex-Specific Prognosis:** Women Patients with differentiated thyroid carcinoma (DTC) present less aggressive disease than men, with less recurrence. Nonetheless, gender disparities in prognosis appear to diminish as the disease progresses to advanced stages, and male and female patients have comparable rates of recurrence in contemporary cohort studies (Moleti et al., 2017; Park et al., 2021).
4. **Epidemiological and Hormonal Aspects:** The rising incidence of PTC among women of childbearing age has led many investigators to speculate that sex hormones are involved in tumorigenesis. Conversely, the more advanced (and aggressive) disease in men could be related to lead and lag times in disease detection and/or inherent biological differences in tumor behavior (Moleti et al., 2017; Douglas et al., 2020).

The significant sex differences in prevalence, pathogenesis, pathology, and prognosis of thyroid cancer underline the importance of including gender as a critical variable in research and therapeutic strategies. Elucidating these differences can allow for personalized therapeutic strategies and better prognostication in the management of thyroid cancer.

1.8.6 Age

The prognostic role of age at the time of diagnosis of thyroid carcinoma is a well-demonstrated factor, especially in papillary thyroid carcinoma (PTC). In the past, age thresholds of 45 years or 55 years have been the rule for determining the assignment to a particular risk group, yet new evidence indicates there may be a continuum of age and prognosis. Older age is closely associated with increased risk of mortality, more aggressive tumor features, and worse clinical outcomes.

Prognostic Relevance of Age Thresholds

- **Historical and Revised Cutoffs**: The AJCC 7th Edition staging system has established 45 years as the breakpoint for risk stratification. However, based on emerging data suggesting declining survival beyond midlife, the 8th Edition increased this threshold to 55 years (Morosán et al., 2022).

- **Continuous Risk Progression:** Studies, including those of Adam et al. (2016), demonstrate that PTC-related death increases in a stepwise manner with increasing age, further supporting the notion that age is a continuous rather than a categorical risk factor.

Tumor Behavior across Age Groups

1. **Older Age and Tumor Aggressiveness**: Patients aged 55 and older more often have high-risk features, such as extrathyroidal extension (ETE), capsular invasion, lymph node metastases (LNM), and higher stage of the disease (Marongiu et al., 2024; Papaioannou et al., 2020).
2. **Thyroid Cancer in Younger Populations**: PTC is the leading thyroid cancer in children and adolescents, with a significant female bias in teenagers (Stenman et al., 2021). In children, including those with HT, the chances of HT progressing to PTC are notably higher, with the prevalence rates for this pathology varying between 0.67–7.87% (Gallant et al., 2023). Pediatric PTC typically presents as a unilateral thyroid nodule and often involves a cervical lymph node (Sur et al., 2020).

Molecular Associations with Age

The BRAF V600E mutation, a necessary PTC driver mutation, is more often identified in older patients. This mutation has been linked to poor clinical outcomes, with higher rates of recurrence and PTC-specific death (Gan et al., 2020; Shen et al., 2018). Its modifying effect by patient age also suggests a joint effect, such as BRAF V600E being compounded with an aging effect on a worse outcome.

Clinical and Staging Implications

- **Prognostic Weight in Staging**: Age is currently the only patient-related factor that the AJCC staging system includes, and itself highlights the significance of age in prognostication of thyroid cancer (Kaliszewski et al., 2020; Kazaure et al., 2018).

- **Recurrence Considerations**: Current staging systems vary as to their emphasis on survival outcome in MTC and, as a consequence, do not comprehensively address the impacts of age on recurrence potential after surgery, particularly among younger patients in whom, despite favorable survival, prevalent regional spread of disease occurs (Tuttle et al., 2019).

Age is one of the most complicated factors in the prognosis of thyroid cancer. Older patients experience higher mortality and aggressive disease behavior, and younger patients, while having good outcomes in general, may demonstrate greater recurring and nodal spread. These complexities underscore the need to evaluate biological/molecular age-associations, including but not limited to BRAF V600E mutation status, to predict prognosis and treatment approaches sensitive to age in patients with thyroid cancer.

1.9 Anatomy and Histological Features of Papillary Thyroid Carcinoma

1.9.1 Gross Morphology

Papillary thyroid carcinoma (PTC) has a widely variable gross appearance and can originate from anywhere in the thyroid gland. Lesions are generally between 2 and 3 cm in size, but size may vary, with some microcarcinomas measuring less than 1 cm. PTC tumors are macroscopically a firm, whitish tumor that often has an infiltrating appearance (Zhao et al., 2022). Tumoral calcification is frequent, and it creates stromal sclerosis, which appears as fibrous scars. The masses are frequently adjacent to the thyroid capsule, and a cystic change sometimes occurs, with some lesions demonstrating nearly complete cystic transformation, which could make radiological and pathological interpretation challenging (Harahap et al., 2024).

1.9.2 Microscopic Features

Histopathologically, PTC is characterised by branching papillae with central fibrovascular cores that are lined by atypical epithelial cells (Pusztaszeri et al., 2023). Their nuclei are particularly striking diagnostically, their nuclei usually being enlarged, elliptical, overlapping, and having a distinctly clear or "Orphan Annie eye" like appearance that is predictive of chromatin distributed

finely and peripheral micronucleoli (Dhingra et al., 2022). Other nuclear characteristic feature includes grooves and intranuclear cytoplasmic inclusions, seen in > 80% (Suzuki et al., 2021).

Psammoma bodies, concentric, laminated calcifications, are typical in the papillae, tumor stroma, or the lymphatic channels (Elsheikh et al., 2024; Rossi et al., 2024). These calcifications are thought to develop from necrosis or infarction of the tumor papillae with subsequent calcification. However, psammoma bodies in lymph nodes are an excellent histological sign of metastatic PTC (Dinets et al., 2024) (Figure 1.10).

1.9.2.1 Patterns of Spread

PTC commonly invades along lymphatic channels with multifocal intrathyroidal involvement and regional lymph node metastasis. More than 50% of patients have nodal metastasis at diagnosis (Wang et al., 2024; Lei et al., 2022). Despite frequent lymphatic invasion, long-term follow-up is favorable, particularly in patients younger than 45 years of age (Wang et al., 2023; Song et al., 2023). Distant metastases, usually to the lungs or bones, are uncommon, found in 2–3% of cases. However, patients may still experience prolonged survival, particularly if the metastases are amenable to treatment with radioactive iodine (Vuong et al., 2022).

The source of multifocality in PTC remains controversial. Although swamp fever and equine leukosis are diseases caused by retroviruses, it is known that retroviral infection is another possible source of T-lymphoma in individual sites of the body, such as the eye gland, consistent with the intraglandular lymphatic metastasis theory, or that it may give rise to multiple independent neoplastic clones. Usually, one predominant lesion with multiple satellite foci favors a lymphatic route of dissemination, whereas many of similar size are suggestive of a multiclonal origin (Zhang et al., 2024; Sun, 2024; Can et al., 2024).

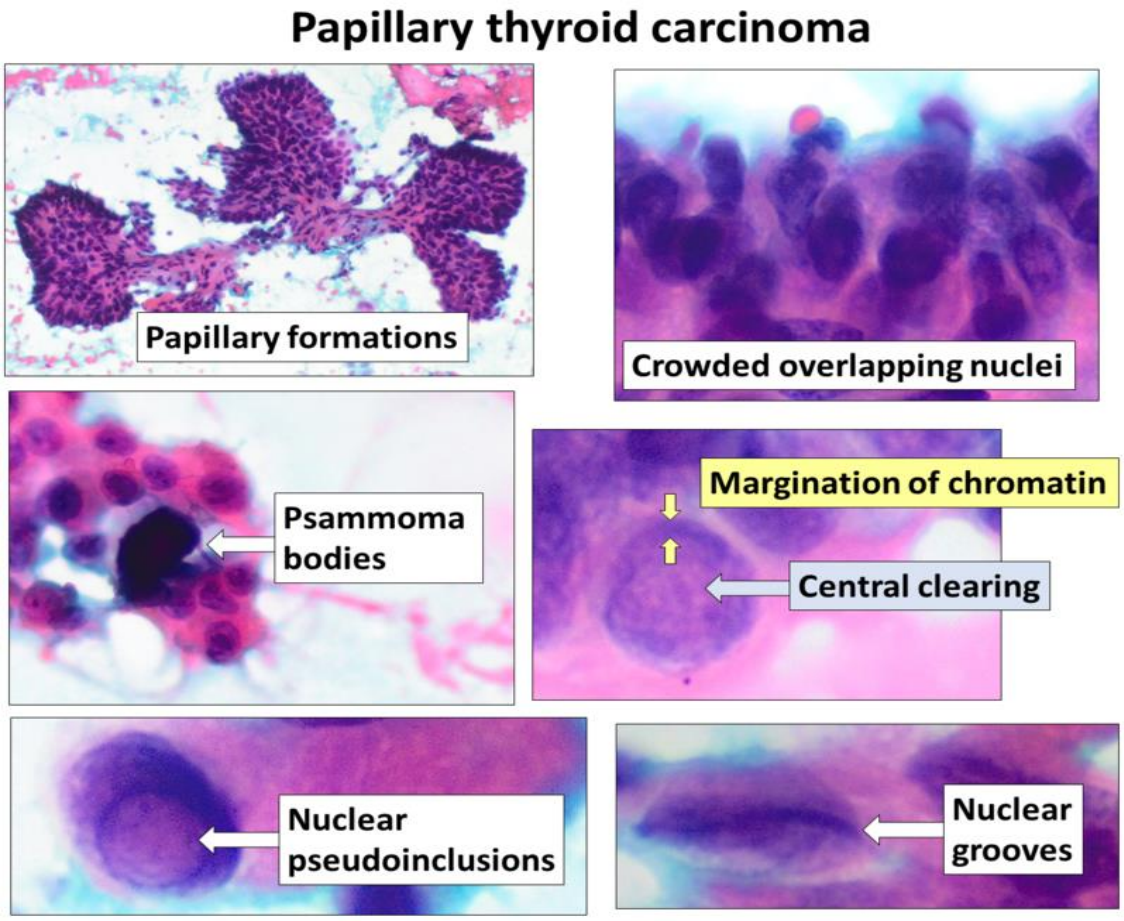


Figure 1.10: Cytopathology of Papillary Thyroid Carcinoma with Pap Stain. (Wikipedia)

1.9.2.2 Cellular and Ultrastructural Characteristics

Cells of PTC are usually cuboidal or columnar and demonstrate papillary and follicular architectural patterns (Lee et al., 2023). The presence of follicles indicates the follicular variant only when the architecture is entirely follicular. When analyzed by electron microscopy, PTC cells have increased numbers of mitochondria, abundant rough endoplasmic reticulum (RER), and prominent apical microvilli, all of which suggest increased metabolism and secretory activity.

1.9.3 Histological Variants of Papillary Thyroid Carcinoma

Papillary thyroid carcinoma (PTC) is a histologically heterogeneous tumor with different types demonstrating unique morphologic characteristics and clinical behaviors (Ravindran et al., 2022):

- **Classic Variant** – This corresponds to the most common type of PTC, which shows classic papillary architecture and typical nuclear features.
- **Follicular Variant** – These tumors primarily have a follicular pattern of growth; however, they still maintain the nuclear features of PTC. The encapsulated noninvasive subtype has been reclassified as Noninvasive Follicular Thyroid Neoplasm with Papillary-like Nuclear Features (NIFTP), because these tumors carry a favorable prognosis with low risk of malignancy.
- **Tall Cell Variant** – Made up of cells that are greater than twice as tall as they are wide, this variant has been shown to display a more aggressive clinical behaviour and poor prognosis.
- **Columnar Cell Variant** – Markedly elongated columnar cells (hyperchromatic nuclei). It tends to be more aggressive and more challenging to treat.
- **Hobnail Variant** – This variant has been described to have micropapillary formation and prominent nuclear atypia, and is associated with a worse prognosis.
- **Diffuse Sclerosing Variant –** Seen in younger patients with lymphocytic infiltration and extensive fibrosis, often with many psammoma bodies, which may reflect a more disseminated disease process.

1.9.4 Papillary Microcarcinoma

Eligible papillary microcarcinomas are defined as PTC tumors with a maximum diameter of 1 cm, which are often coincidentally detected through surgery or based on autopsy reports (Ma et al., 2021). While most of these tumors have an indolent behavior, a subset can have aggressive behavior, supporting the importance of a personalized clinical evaluation. The term “occult papillary tumor” has been proposed to reduce undue psychological impact on telling a patient their diagnosis (Chin et al., 2023).

1.9.5 Encapsulation and Invasion Patterns of PTC

The majority of PTC lesions are not encapsulated and tend to metastasize via lymphatics rather than the bloodstream. Metastases to regional lymph nodes may infrequently undergo cystic change, which may be confusing with limited numbers of malignant cells present. Distant metastatic disease, especially to the lungs, may radiologically appear as multiple discrete nodules or, in rare cases, a diffusely spread snowflake-like pattern (Akbulut et al., 2021).

Papillary thyroid carcinoma (PTC) is a specific malignant tumor that has characteristic gross, histologic, and cytologic findings. Its typical nuclear appearance, penchant for lymphatic spread, and often multicentric nature are the hallmark features for diagnosis. Although the disease carries an overall favorable prognosis, particularly in young patients, specific aggressive variants may require close observation and more aggressive therapeutic measures to achieve better clinical outcomes.

1.10 Molecular Mechanisms Underlying Papillary Thyroid Carcinoma (PTC)

Papillary thyroid cancer (PTC), the most common type of thyroid cancer, develops as a result of consecutive genetic lesions that constitutively activate oncogenic signaling pathways. Understanding this molecular event is crucial for designing targeted therapies and improving clinical decision-making.

1.10.1 Activation of the MAPK/ERK Signaling Cascade

A central molecular hallmark of PTC is the abnormal activation of the MAPK/ERK pathway, which is mainly driven by mutations in regulatory genes (Figure 1.11). The BRAF V600E mutation, identified in 40–60% of PTC patients, constitutively activates the MAPK pathway, leading to uncontrolled cellular proliferation and differentiation, as well as tumor survival (Wei et al., 2022). This mutation is closely correlated with poor clinical outcomes, including extrathyroidal extension, lymph node metastasis, and poor response to radioactive iodine (Zhang et al., 2024).

The MAPK pathway is also essential for cell cycle control, differentiation, and survival. In PTC, its overactivation is frequently linked to genetic aberrations, including BRAF V600E and RAS mutations (including NRAS, HRAS, and KRAS) and gene rearrangements such as RET/PTC fusions (Musholt et al., 2019). Furthermore, ALK mutations have been described as infrequent and consequently relevant contributors to PTC oncogenesis (Murugan et al., 2021).

1.10.1.1 Oncogenic Signaling and Downstream Molecular Effects

Aberrancy in the MAPK pathway triggers a downstream signaling network that, in turn, promotes tumor growth and progression through various molecular mechanisms. These are, for example, complete epigenetic resetting (e.g., global hypomethylation and hypermethylation) and upregulation of proteins derived from traits convenient to tumors (Marczyk et al., 2023). Key upregulated factors include:

- **Angiogenesis-promoting molecules**: Vascular endothelial growth factor A (VEGFA) (Razy et al., 2019)
- **Extracellular matrix (ECM) remodeling enzymes**: Matrix metalloproteinases (MMPs), urokinase-type plasminogen activator (uPA), and its receptor (uPAR) (Piperigkou et al., 2021)
- **Inflammatory cytokines**: Nuclear factor-κB (NF-κB), transforming growth factor-β1 (TGFβ1) (Mojsilovic et al., 2023)
- **Cell adhesion and migration markers**: Thrombospondin-1 (TSP1), prokineticin-1 (PROK1), prohibitin, and vimentin (Shafabakhsh et al., 2023)

These mediators collaborate to promote tumor cell survival, invasion, metastasis, and the formation of new blood vessels.

1.10.1.2 Tumor Microenvironment and ECM Remodeling in PTC

The tumor microenvironment (TME) is a dynamic factor in PTC development. Formerly considered an inert substance, the extracellular matrix (ECM) has been redefined as a biologically active matrix affecting tumor biology. Oncogenic mutations, such as the BRAF V600E mutation, dictate ECM composition and remodeling, leading to tumor potency:

- **BRAF V600E** promotes TSP1 secretion, which binds to integrins, cytokines, growth factors, such as VEGFA, MMPs, etc, contributing to increase malignancy (Melaccio et al., 2021).
- **TGFβ1**, when released by MAPK activation, supports ECM remodeling and the establishment of an inflammatory and pro-tumorigenic milieu (Jarboe et al., 2021).
- **Reactive oxygen species (ROS)** produced from inflammatory signals may subsequently activate MAPK signaling, thus maintaining an oncogenic loop (Eng et al., 2023).

1.10.1.3 Contribution of Stromal Cells in Tumor Progression

In particular, non-cancerous stromal cells in the TME, such as tumor-associated macrophages (TAMs) and cancer-associated fibroblasts (CAFs), are essential regulators of PTC progression. TAMs promote inflammation via TGFβ1 expression, and CAFs regulate tumour dynamics via FGFR2 activation. It is interesting to note that the FGFR2 isoforms – FGFR2-IIIb (in tumor cells) and FGFR2-IIIc (in fibroblasts), which are differentially expressed, can also mediate tumor–stroma interaction and can lead to a faster transition into a malignant stage (Tao et al., 2023; Xu et al., 2022).

1.10.1.4 MAPK Signaling Crosstalk with Other Oncogenic Pathways

Outside of the MAPK pathway, PTC pathogenesis is influenced by crosstalk with other signaling pathways:

- **NF-κB Pathway**: Upregulation of the NF-κB signaling, frequently associated with BRAF V600E, results in the transcription of genes involved in inflammatory response, anti-apoptosis, and invasion. This represents a further level of oncogenic addiction (Crescenzi et al., 2024).
- **FOXO3 Suppression**: During normal conditions, activation of the tumor suppressor FOXO3 is conducted by RASSF1A-MST1 and tends to cause apoptosis. Precise data demonstrate that BRAF V600E also enters this pathway but disrupts it, sending it in the wrong direction to diminish apoptosis and favor tumor survival (Yu et al., 2024).

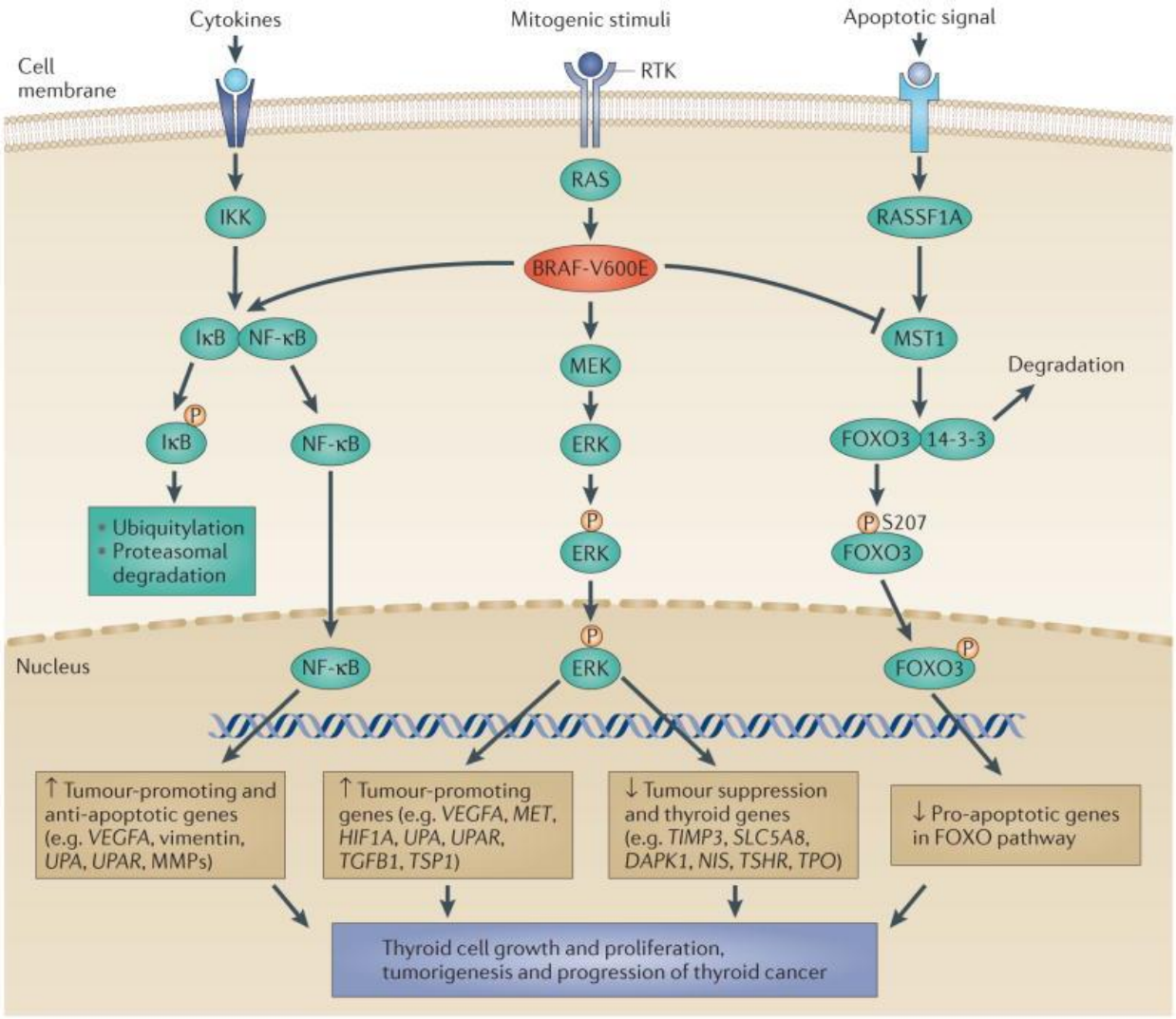


Figure 1.11: The MAPK Pathway and Its Associated Signaling Networks in Thyroid Cancer. (Mingzhao Xing; 2013)

PTC originates from an intricate interplay of molecules, the most important of which is the MAPK/ERK pathway. Genetic and epigenetic mutations, as well as alterations in the TME, cooperate in cancer development. Cross-talk between MAPK and its downstream targets, such as NF-kB, FOXO3, etc., increases tumour aggressiveness, immunoevasion, and chemo- and radio-therapy resistance. Such findings suggest potential targets for focused therapy, including MAPK inhibitors, extracellular matrix (ECM)/tumor microenvironment (TME) targeting agents, and immunological manipulation of the tumor-stromal dialogue.

1.10.2 RET/PTC Rearrangements

The most common genetic lesions in the papillary thyroid carcinoma (PTC) are represented by RET/PTC rearrangements, particularly those occurring in radiation-induced and pediatric tumors. These rearrangements consist of the RET proto-oncogene's tyrosine kinase domain fused to the 5′ sequences of several partner genes, resulting in constitutive activation of RET signaling cascades. Over 15 RET/PTC fusion types have been described, with RET/PTC1 and RET/PTC3 being the most commonly observed forms (Elisei et al., 2023).

These fusions constitutively activate the RET kinase in a ligand-independent manner and subsequently lead to activation of oncogenic signaling pathways that result in cellular proliferation and resistance to apoptosis (Ullmann et al., 2022). Tumors harboring RET/PTC are typically of classic papillary morphology and present as a tumor in younger patients, especially those with a history of exposure to ionizing radiation (Soares et al., 2021).

The heterogeneous nature of the RET/PTC fusions in multifocal PTC cases also indicates that, rather than occurring at late stages of tumorigenesis, these genetic events must be independently acquired in a neoplastic-prone thyroid gland, thus backing the idea that RET/PTC may be an early driver in tumor initiation rather than in progression (Hu et al. 2021).

In summary, RET/PTC rearrangements have a significant role in the early molecular pathogenesis of PTC, particularly in pediatric and radiation crisis-related cases. Their discovery highlights the importance of chromosomal rearrangements in thyroid tumourigenesis and suggests new molecular targets for improved diagnosis and early treatment of thyroid tumors.

1.10.3 RAS Mutations

The RAS oncogene family, including HRAS, KRAS, and NRAS, is mutated in 10–20% of PTCs, although at an incidence five times higher in the follicular variant. These mutations allow the dysregulated activation of the MAPK and PI3K/AKT signaling pathways, leading to tumorigenesis (Haghzad et al., 2024).

RAS-mutant PTCs tend to display a less aggressive clinical behavior compared to BRAF-mutant tumors. They generally have a more indolent disease course and lower rates of recurrence and metastasis, and are sometimes more responsive to traditional therapies (Boucai et al., 2022).

Translational relevance: Due to their inherent dual-activation potential, RAS mutations represent an intersection of the primary signaling pathways that drive PTC, underscoring their importance in thyroid cancer biology.

1.10.4 PI3K/AKT/mTOR Pathway

While the MAPK pathway is the predominant force in PTC development, evidence has emerged suggesting the importance of the PI3K/AKT/mTOR axis in tumor growth and carcinogenesis. Genetic mutations in this pathway (PIK3CA, AKT1, PTEN) are more frequent in aggressive, poorly differentiated, and therapy-resistant PTC (Pappa et al., 2021).

PI3K/AKT signaling has been constitutively activated in a variety of oncogenic processes, leading to increased cell proliferation, inhibition of apoptosis, metabolic reprogramming, and chemotherapy resistance (Fig. 1.12). Such molecular changes can occur by means of direct gene mutations or by interaction with other oncogenic signaling pathways (Crezee et al., 2020).

Notably, PI3K/AKT/mTOR pathway activation has been frequently associated with poor clinical-pathological factors, high tumor aggressiveness, and poor survival (Liu et al., 2024). This has led to the pathway being established as an attractive potential target for directed therapy, and several inhibitors are currently being evaluated for advanced or refractory thyroid carcinoma. The PI3K/AKT/mTOR signaling pathway is the second-most-important molecular driver in PTC, playing a significant role in disease aggravation and treatment resistance.

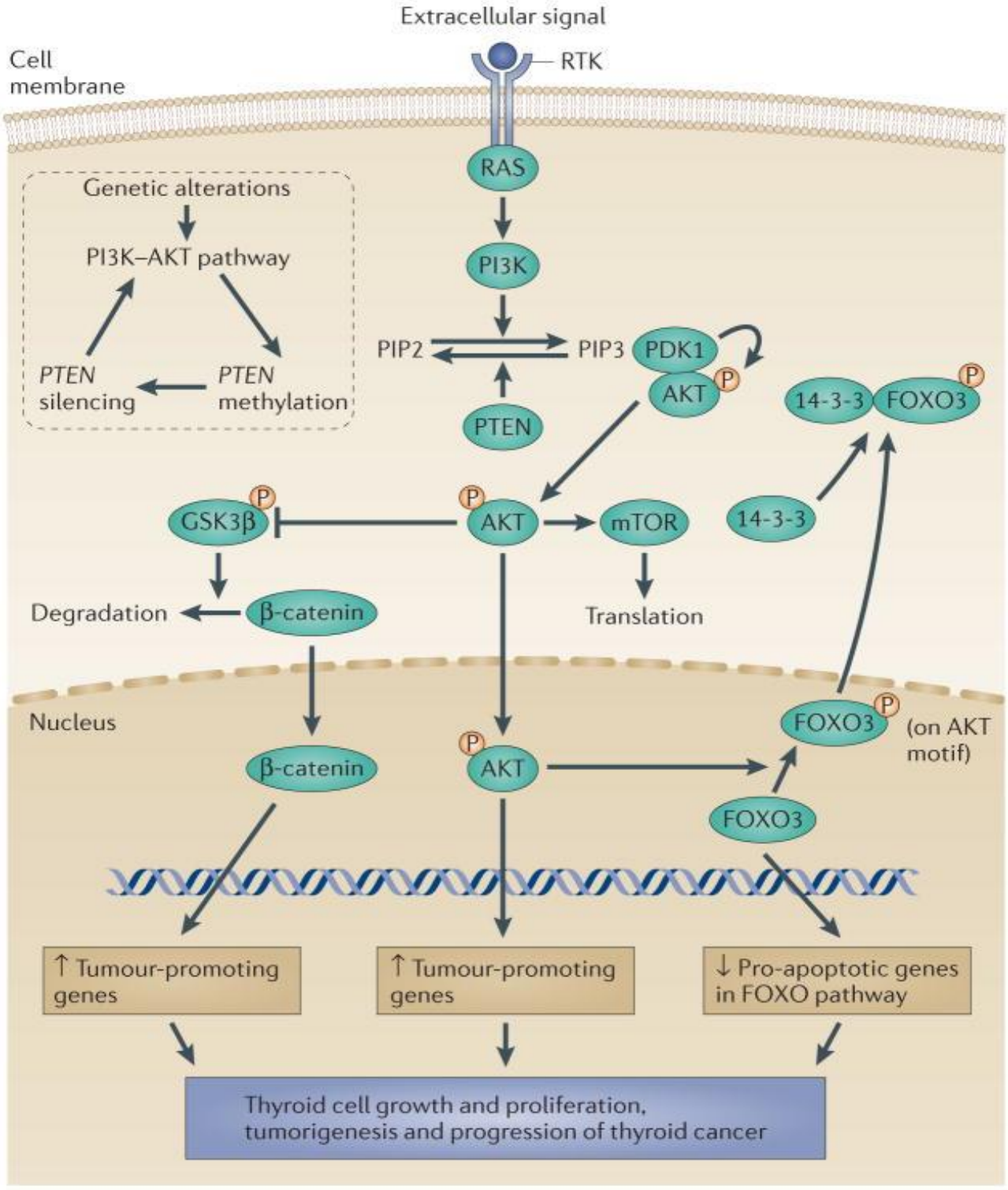


Figure 1.12: The PI3K–AKT Pathway and Its Associated Signaling Networks in Thyroid Cancer. (Mingzhao Xing; 2013)

1.10.5 Interplay between MAPK and PI3K–AKT Pathways in Advanced Disease

There is crosstalk between dedifferentiated and anaplastic forms, in which both MAPK and PI3K–AKT pathways are concomitantly activated during thyroid tumorigenesis. It has been well established that 80-81% of cases of ATC have concurrent activation of both these signaling pathways, which is consistent with the highly aggressive nature of this cancer type (Pozdeyev et al., 2020; Zeng et al., 2024). This dual activation is reportedly less common in well-differentiated thyroid cancers, for instance, follicular thyroid adenocarcinoma (FTA). [Google Scholar]) However, its incidence is notably higher in PDTC and ATC, attesting its key role in malignant progression (Cabanillas et al., 2023).

Immunohistochemistry studies showed a common co-expression of phospho-ERK and phospho-AKT proteins in ATCs, indicative of concurrent MAPK and PI3K–AKT pathway activation, unlike that observed in well-to-moderately differentiated thyroid carcinomas (Lasolle et al., 2024). This is strongly supported by mouse studies in which loss of PTEN and activation of KRAS cooperatively drive aggressive thyroid tumorigenesis. In contrast, the stimulation of either pathway alone does not (Haghzad et al., 2024). Similar findings have been proven in melanoma, where high-metastatic disease develops after BRAFV600E expression is combined with PTEN loss, further emphasizing the transferability of this dual-pathway model (Florent et al., 2023).

1.10.6 Proposed Model of Thyroid Cancer Evolution

The molecular pathogenesis of thyroid cancer can be conceptually divided into distinct categories based on the prevailing signaling defects. Mutations in the MAPK pathway, including BRAFV600E and RET/PTC rearrangements, are major drivers of PTC, whereas alterations in the PI3K–AKT pathway, including RAS and PIK3CA mutations as well as PTEN loss, prevail in FTA and FTC (Wang et al., 2023; Rangel-Pozzo et al., 2020). As disease develops, these pathways intersect, and co-activations foster PDTC. Additional drivers, such as TP53, CTNNB1, and ALK (Cirello et al., 2024; Singh et al., 2021) mutations, drive the progression to full-blown ATC, the most severe subtype of thyroid cancer.

Additionally, ATC can arise de novo or can progress from underlying PTC or FTC lesions as key mutations accumulate. Other oncogenic drivers, such as loss of epigenetic control or activation of secondary signaling cascades (e.g., Wnt–β–catenin, NF-κB, and HIF-1α), promote further tumor progression and dedifferentiation (Zhang et al., 2023). This integrative paradigm highlights thyroid cancer as a dynamic process of multistep evolution, where the sequential accumulation of "hits"—of both genetic and epigenetic nature—underlies the passage from indolent to aggressive clinical disease (Figure 1.13).

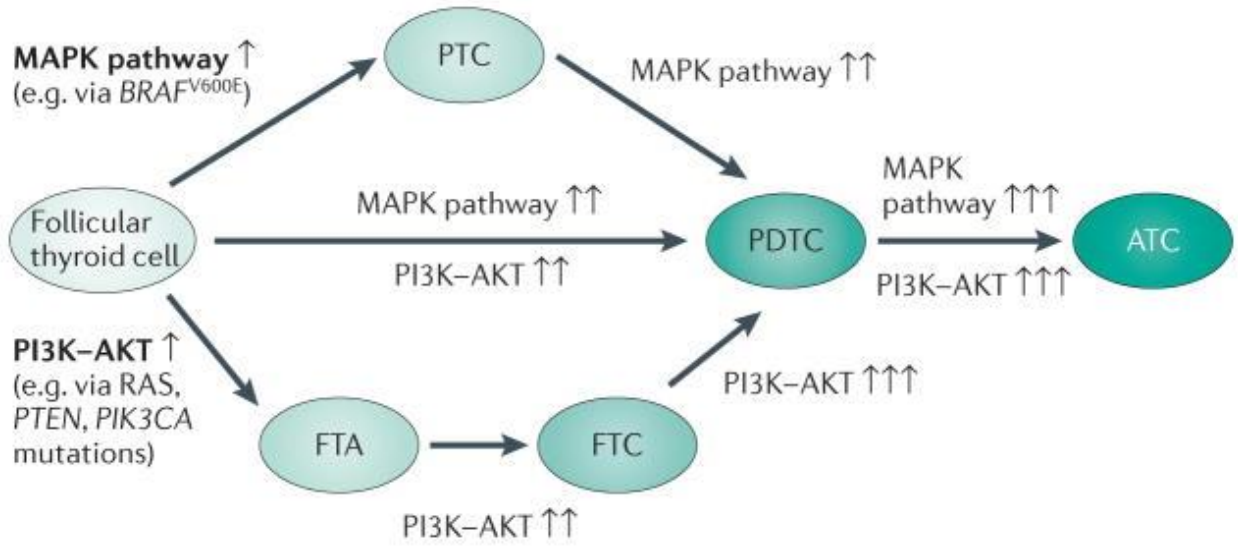


Figure 1.13. A Model Illustrating Thyroid Tumorigenesis Progression Driven by the MAPK and PI3K–AKT Pathways. (Mingzhao Xing; 2013)

1.10.7 TERT Promoter Mutations

Mutations in the telomerase reverse transcriptase (TERT) gene promoter are detected in about 10–15% of papillary thyroid carcinoma (PTC) cases and are associated with an aggressive phenotype, including a high incidence of tumor recurrence and shorter overall survival (Sako et al., 2024). These mutations increase telomerase activity, allowing continued proliferation and cellular immortality — key characteristics of cancer.

TERT promoter mutations coexisting with the BRAF V600E mutation are associated with greater tumor aggressiveness. It is a strong predictor of poor clinical outcomes, making this molecular combination a highly predictive indicator of high-risk disease (Gerwen et al., 2023).

Thus, the TERT promoter mutations are prognostically advantageous and potential molecular markers for the sub-classification of patients with higher-grade disease.

1.10.8 Epigenetic Alterations

In addition to genetic mutations, epigenetic changes, including DNA methylation, histone modifications, and microRNA (miRNA) dysregulation, play crucial roles in PTC pathogenesis. It is worth mentioning that the hypermethylation of tumor suppressor genes, including RASSF1A and SLC5A8, has been extensively observed, leading to their transcriptional repression and loss of the tumor-suppressive capacity (Acuña et al., 2023).

At that time, dysregulated expression of miRNAs, mainly those of the miR-146b and miR-221 families, plays an essential role in regulating cell proliferation, migration, and resistance to apoptosis by modulating major oncogenic and tumor suppressor pathways (Galuppini et al., 2022).

Such epigenetic alterations not only associate with tumorigenesis and progression but also present potential for diagnostic and therapeutic applications.

1.10.9 Immune Microenvironment

Regulatory immune cells, particularly tumor-associated macrophages (TAMs) and regulatory T cells (Tregs), play an essential role in creating an immune-suppressive microenvironment that enables immune evasion and tumor progression (Song et al., 2023). High levels of TAMs are associated with lymph node metastasis and later stages of disease, indicating their involvement in promoting tumor aggressiveness and metastasis (Katoh et al., 2024).

Awareness of immunological mechanisms in the PTC microenvironment is enlightening regarding the tumor's behavior. It opens a door for immunotherapies that can manipulate immune cell functions to suppress tumor growth.

1.11 Metastatic Behavior of Papillary Thyroid Carcinoma (PTC)

PTC is the most common type of thyroid cancer and typically has an excellent prognosis. However, a select group of patients has a more aggressive form of the disease, characterized by regional and distant spread, which negatively impacts the disease course and treatment (Figure 1.14).

1.11.1 Regional (Lymph Node) Metastasis

The rate of lymph node metastases in PTC is between 30% and 50%, and most commonly they occur in the central and lateral compartments of the cervical lymphatic chain (Yu et al., 2024). While nodal metastasis may not adversely affect survival in low-risk groups, it does increase the risk of local and regional recurrence. In contrast, among high-risk patients (extranodal extension, bulky nodal disease), the outcomes are much worse (Kang et al., 2023; Stenman et al., 2022). Diagnostic imaging, in particular high-resolution ultrasound, often depicts microcalcifications and cystic metamorphosis in lymph nodes, which strongly indicate metastatic dissemination (Xue et al., 2021).

The tumor microenvironment is pivotal in promoting nodal spread. CAFs and TAMs secrete pro-tumorigenic cytokines and growth factors, which potentiate the invasive ability of cancer cells (Febrero et al., 2024). Furthermore, high stromal fibrosis at the tumor–host interface has been associated with a higher risk of lymphatic invasion (Avagliano et al., 2022).

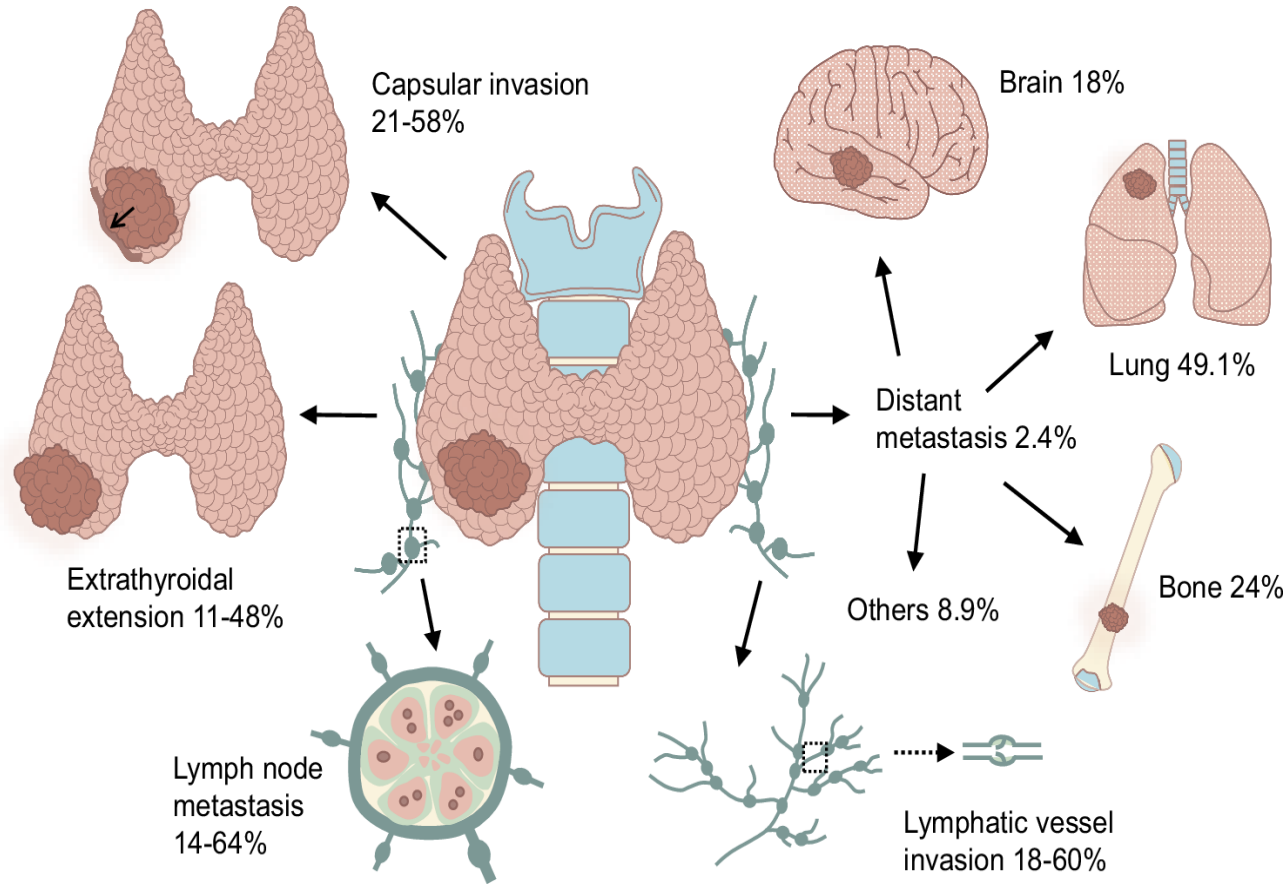


Figure 1.14: Metastatic Traits of PTC. (Zhang et al., 2024)

1.11.2 Distant Metastasis

In about 5–10% of PTC cases, distant metastases occur, with the most common target organs being the lungs, followed by bone, liver, and brain (Toraih et al., 2021). Pulmonary involvement may manifest in an indolent, micro-nodular pattern or as a more aggressive, macro-nodular pattern, having poor prognostic impact (Chua et al., 2024). Certain genetic mutations, such as TERT promoter and BRAF V600E, are strongly correlated with increased metastatic potential (Landa et al., 2023). Survival rates are highly variable, with 5-year survival rates for patients with distant metastases ranging from 24% to 76%, depending on the number of organs involved and the effectiveness of therapy. The presence of multi-organ metastasis or brain disease is also correlated with a much worse prognosis (Toraih et al., 2021).

1.11.3 Molecular Basis of PTC Metastasis

The metastatic capability of PTC is driven by molecular aberrations, mainly involving the MAPK and PI3K–AKT cascades, which promote cellular motility, invasiveness, and survival (Zhang et al., 2024). The BRAF V600E mutation is widely associated with adverse clinicopathologic characteristics, including extrathyroidal extension, nodal metastasis, and treatment refractoriness. In addition to these genetic drivers, epigenetic mechanisms, including promoter hypermethylation and histone modifications that repress genes key to cell adhesion, extracellular matrix degradation, and angiogenesis, are also involved (Hu et al., 2021). For example, hypermethylation of the TIMP3 promoter has been linked to enhanced invasiveness and metastatic potential in tumors (Sabi et al., 2024).

Although PTC frequently follows a slow and favourable course, the development of metastases requires vigilance and personalized therapeutic management. Given the complex interplay between genetic alterations, epigenetic modifications, and the tumor microenvironment, it is of fundamental importance to develop targeted therapies to control advanced PTC.

1.12 Clinical Manifestations of Thyroid Cancer

Symptoms Thyroid cancer frequently does not cause any symptoms in early stages, and only in later stages do they appear. Symptoms vary depending on the type of cancer, the size of the tumor, and the extent to which it has spread locally or to other parts of the body (NHS, 2024; Mayo Clinic, 2024). Although some features are indistinguishable from benign thyroid diseases, patients with signs or symptoms that persist or worsen after initiation of synthetic levothyroxine replacement should be thoroughly evaluated for the earliest possible diagnosis and targeted treatment

1.12.1 Common Clinical Signs

- **Often asymptomatic in the early stages:** Frequently asymptomatic at first: Thyroid cancer is often discovered by chance during a check-up, or other unrelated tests and scans, as patients don’t usually have early symptoms (Song et al., 2024).

- **Neck lump/swelling:** A steadily increasing, non-tender, firm nodule in the anterior neck area that is not easily moved is a typical clinical feature of cancer (Chowdhury et al., 2024); this is commonly referred to as an open neck mass or swelling.
- **Voice alterations or hoarseness:** Pressure from the tumor on, or interference with, the recurrent laryngeal nerve may alter the voice or cause persistent hoarseness (Cipolla et al., 2021).
- **Swallowing difficulties (dysphagia):** As the tumor grows, it can compress the esophagus, causing pain or a blocked sensation during swallowing (Santa Maria et al., 2024).
- **Chronic cough:** A long-lasting cough, particularly in the absence of an infection or allergy, may indicate a progressing tumor (Sun et al., 2024).

1.12.2 Advanced and Progressive Symptoms

- **Localized pain:** Tumor involvement of adjacent structures such as the jaw, ear, and neck due to pain reflects local advancement of tumor into surrounding structures (Chowdhury et al., 2024).
- **Cervical adenopathy:** The most frequent finding in the late stages is enlargement of the cervical lymph nodes due to metastatic spread (Ji et al., 2023).
- **Difficulty breathing:** Compression from a tracheal tumor may cause difficulty in breathing, shortness of breath, and a feeling of choking (Kinuya et al., 2006).
- **Facial flushing:** There may be episodic facial flushes in medullary thyroid carcinoma (MTC) secondary to the release of calcitonin-related peptides, especially in those with light skin (Cohen et al., 2023).
- **Gastrointestinal Symptoms:** MTC-associated hormonal hypersecretion can induce bowel movement changes, exceptionally prolonged diarrhea (Mosiychuk et al., 2021).
- **Systemic symptoms:** Unintentional weight loss, fatigue, and general malaise can occur with advanced or metastatic disease (De Leo et al., 2021).

As benign and malignant thyroid diseases overlap considerably, the presence, persistence, or worsening of symptoms should all prompt comprehensive clinical and instrumental investigation. Due to the difficulty of detection, early detection is essential for a better prognosis and treatment.

1.13 Thyroid Cancer Diagnosis

The diagnosis of thyroid cancer is multifaceted, which comprises clinical assessment, imaging modalities, laboratory studies, and cytological examination. The prompt and accurate diagnosis is of paramount importance for an appropriate therapeutic approach and a better prognosis.

1.13.1 Clinical Assessment

Diagnosis The diagnosis of thyroid cancer starts with a complete clinical assessment, including a full medical history and a neck examination in search of palpable thyroid nodules or lymphadenopathy (Grani et al., 2024). Major risk factors include a history of previous exposure to radiation, a family history of thyroid malignancy, and symptoms that are indicative of thyroid problems. A detailed record of these factors is crucial for accurate risk estimation (Berinde et al., 2022).

1.13.2 Imaging Techniques

- **Ultrasonography:** High-resolution neck ultrasound is the imaging modality of choice for thyroid nodules. It describes characteristics of a nodule, including its size, internal composition, echogenicity, margin, and microcalcification (Ghai et al., 2024). Suspicious features such as hypoechogenicity, irregular shape, microcalcifications, and a taller-than-wide shape are more frequently associated with cancer (Russell et al., 2022). The American College of Radiology's TIRADS classification is frequently used to assess the risk of malignancy and guide fine-needle aspiration (Strieder et al., 2022).
- **CT and MRI:** Despite not often being used as a primary diagnostic approach, CT and MRI may be useful for the evaluation of large tumors, in particular regarding local invasion and distant spread (Giovanella et al., 2022). Administering iodinated contrast in conjunction with CT should be carefully weighed, because it may interfere with subsequent radioiodine therapy (Alabousi et al., 2022).

- **Thyroid Scintigraphy**: The imaging method by means of radiotracers, like technetium-99m or iodine-123, to assess the morphology and functioning of the thyroid (Trimboli et al Investigator 2024). It helps differentiate hyperfunctioning ("hot") nodules, mostly benign, from hypofunctioning ("cold") ones, which are more often malignant (Giovanella et al., 2022).
- **SPECT/CT:** The integration of functional and anatomical imaging in SPECT/CT offers better localisation and characterisation of abnormal thyroid tissues. It is especially helpful in locating ectopic thyroid tissue, in detecting metastasis, and in writing on the remaining post-surgical tissue (Pește an et al., 2024).
- **PET/CT:** PET/CT using 18F-FDG is useful to detect recurrence or metastases of thyroid cancer, especially in those patients with increased thyroglobulin but with negative radioiodine scanning. It is also useful for identifying metabolically active aggressive subtypes, such as poorly differentiated and anaplastic thyroid cancer (Zampella et al., 2021).

1.13.3 Laboratory Evaluation

- **Thyroid Function Tests:** The primary test for thyroid cancer is a TSH level. Most patients are euthyroid, although changes in TSH may serve as a hint to other coexisting functional thyroid diseases (Andersen et al., 2022; Haymart et al., 2021).
- **Calcitonin and CEA:** Medullary thyroid carcinoma has as one of its tumor markers increased serum calcitonin, and CEA serves as an additional tumor marker (Wang et al., 2024).

1.13.4 Fine-Needle Aspiration Biopsy (FNAB)

Ultrasound-guided FNAB is considered the gold standard for evaluating thyroid nodules (Tarigan et al., 2022). It includes cellular aspiration for cytologic examination. The Bethesda System characterizes the results in six diagnostic categories and provides malignancy risk and management recommendations based on the results (Ali et al., 2023):

1. Non-diagnostic/Unsatisfactory

2. Benign
3. Atypia or Follicular Lesion of Undetermined Significance (AUS/FLUS)
4. Follicular Neoplasm or Suspicion for Follicular Neoplasm
5. Suspicion of Malignancy
6. Malignant

These categories guide clinical decisions ranging from observation to surgical intervention.

1.13.5 Molecular Diagnostics

For inconclusive results following FNAB, one can perform molecular analyses for mutations (e.g., BRAF, RAS, RET/PTC, PAX8/PPARγ) to help stratify the risk of malignancy and guide therapy (Patel et al., 2023; Kannan et al., 2022). This molecular understanding enhances diagnostic accuracy and assists in individualized treatment approaches.

1.13.6 Emerging Technologies in Diagnosis

Novel diagnostic methods, such as radiomics and artificial intelligence (AI), are being explored to enhance diagnostic power. Radiomics derives image-based features, and AI models can analyze these features to enhance the discrimination between benign and malignant nodules. These advances are expected to facilitate more precise and individualized diagnostics (Toro-Tobon et al., 2023; Habchi et al., 2023).

The diagnosis of thyroid cancer is an integrated approach that includes clinical decision-making, state-of-the-art imaging, biochemical evaluation, cytology, and molecular genotyping. Continued technological development will enhance diagnostic roadmaps and potentially improve patient outcomes through precision medicine.

1.14 Thyroid Cancer Management Approaches

Therapy of thyroid cancer is a very individualized process, taking into account tumor histology, staging, and molecular profile. A multidisciplinary treatment approach is often required and

includes surgery, RAI therapy, TSH suppression, external beam radiation therapy (EBRT), and targeted therapies toward altered molecular markers. For the most common type of DTC (papillary and follicular), surgery remains the standard of care. On the other hand, more aggressive variants, such as ATC and MTC, require global cases to improve patients' survival (Prete et al., 2024).

1.14.1 Surgical Treatment Strategies

Surgical resection is still considered the first choice of treatment for most thyroid cancers, especially for the DTC types like the papillary and follicular carcinomas (Stewart et al., 2021). Surgical modalities are basically standard cervicotomy and laparoscopic approaches, consisting of lobectomy and total thyroidectomy, dictated by the size of the tumor, anatomical spread, and lymph nodal involvement. Total thyroidectomy is the treatment of choice for lesions>4 cm, extracapsular extension, or clinically positive lymphatic metastases (Xu et al., 2023). In cases involving lymph node involvement, central or lateral neck dissection may be required (Agcaoglu et al., 2024; Lo et al., 2022). For smaller and lower-risk tumors, such as papillary microcarcinomas, lobectomy may be sufficient (Bi et al., 2023). Robotic-assisted thyroidectomy. This technique, subject to ongoing technological advancements, uses a robotic system to provide minimally invasive access, a short hospital stay, and a lower complication rate, and represents an attractive alternative for selected patients (Park et al., 2024).

1.14.2 Radioactive Iodine (RAI) Therapy

RAI therapy is an essential adjuvant for DTC after thyroidectomy, focusing mainly on residual thyroid tissue or metastases, and is administered with iodine-131 (Valerio et al., 2023). This is especially useful in the case of large high-risk tumors with involvement of the local tissues or distant metastasis (Mayson et al., 2021). But in low-risk patients, RAI is generally not indicated, and the therapeutic advantage is lacking (Zhao et al., 2022). Tailored dosing schedules are being investigated to improve drug effectiveness and reduce toxicity (Pacilio et al., 2022).

1.14.3 Thyroid Hormone Suppression Therapy

Postoperative thyroid hormone treatment is required in the treatment of DTC to suppress TSH and prevent recurrence. Levothyroxine (LT4) is given lifelong to maintain euthyroidism and to minimize TSH-stimulated growth of any residual tumor (Miccoli et al., 2021). The degree of TSH suppression is classified by risk of recurrence: TSH is severely suppressed (below 0.1 mU/L) in high-risk patients and moderately suppressed (0.5–2.0 mU/L) in low-risk patients (Giovanella et al., 2023; Lee et al., 2024). Current studies aim to individualize the LT4 dosing regimens to achieve oncological benefits and prevent long-term complications, such as osteoporosis and cardiovascular disease (Silaghi et al., 2022; Yang et al., 2022; Ku et al., 2021).

1.14.4 Molecular Targeted Therapies in Advanced Thyroid Cancer

Targeted therapies play a crucial role in the management of advanced thyroid cancer by targeting abnormal molecular pathways involved in tumor growth and survival (Zhang et al., 2023). Among these therapies are the tyrosine kinase inhibitors (TKIs) lenvatinib and sorafenib, which inhibit tumor proliferation and angiogenesis, as well as inhibitors of the inappropriately activated MAPK and PI3K-AKT pathways (Kędzierska et al., 2024; Fallahi et al., 2022). Patients with RAI-refractory DTC and notably patients with poorly differentiated and anaplastic features derive the greatest benefit from these therapies (Prete et al., 2021). In these populations, TKIs such as lenvatinib and sorafenib have significantly increased progression-free survival (Kim et al., 2023; Karapanou et al., 2022). In addition, more targeted options are now also available for patients with RET gene rearrangements, including selective RET inhibitors such as selpercatinib (Hamidi et al., 2024; Román-Gil et al., 2022).

1.14.5 Immunotherapeutic Approaches

Immunotherapy is emerging as a treatment option for advanced thyroid cancers, particularly those unresponsive to conventional treatment options including surgery, RAI, and TKIs (Song et al.,

2024). This strategy leverages the immune system to identify and kill tumour cells, usually with immune checkpoint inhibitors. Medications such as pembrolizumab and nivolumab—anti-PD-1 monoclonal antibodies—restore immunosurveillance by interfering with the PD-1/PD-L1 axis (Chera et al., 2022; Pinheiro et al., 2024). These therapies have shown hopeful outcomes in more aggressive forms of ATC (Shobab et al., 2024; Zhu et al., 2024). Current clinical trials report the combination of checkpoint inhibitors with TKIs to overcome resistance and improve treatment response (Kobayashi et al., 2024; Gao et al., 2023; Ragusa et al., 2022). While still experimental, immunotherapy appears to be a promising therapeutic option in refractory cases or for patients with metastatic disease, provided that additional predictive biomarkers and the optimal treatment regimen are identified.

1.14.6 Role of Chemotherapy

Chemotherapy, using also cytotoxic drugs to stunt the division of cancer cells, has a restricted, but targeted, role in the management of thyroid carcinoma (Behranvand et al., 2022). Even though DTC is usually sensitive to RAI and targeted agents, chemotherapy could be considered in the refractory setting or in extremely aggressive histologies such as ATC that are generally refractory to all treatment approaches (Jannin et al., 2022). Standard chemotherapies, such as doxorubicin, cisplatin, and paclitaxel, have been administered in both palliative and multimodal settings, often in combination with radiation and immunotherapies (Mirzaei et al., 2023; Pavlidis et al., 2023). For instance, paclitaxel is beneficial when combined with targeted or immune-based treatments (Yang et al., 2021). Nevertheless, chemotherapy, because of its systemic toxicity and small survival benefit, is recommended only for patients with rapidly progressive courses (Mathur et al., 2022).

Novel therapies, such as TKIs (sorafenib, lenvatinib), RET inhibitors (pralsetinib), and PD-1 immunotherapies, have reshaped the treatment landscape for advanced or RAI-refractory thyroid cancers (Su et al., 2024; Turner et al., 2024; Chen et al., 2024). Studies on efficient drug combinations and molecular markers that tailor treatments to individual patients are being pursued (Bhattacharya et al., 2023; Shi et al., 2023).

1.15 Recent Advances in Blood-Based Biomarkers for Thyroid Cancer Detection and Prognostication

The identification, prognosis, and treatment of thyroid cancer primarily rely on imaging, histology, and blood markers (Das et al., 2023). These biomarkers are crucial for assessing thyroid gland function and cancer-related molecular signaling, as well as for monitoring disease progression or relapse (Chen et al., 2024; Belousov, 2022).

1.15.1 Conventional Thyroid Function Tests and Their Diagnostic Constraints

Thyroid function is regularly evaluated by standard tests, including thyroid-stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4) (Yazdaan et al., 2023). Although thyroid hormones are useful for assessing endocrine activity, they often remain within the reference range in cases of thyroid cancer, limiting their ability to diagnose it (Van et al., 2023; Andersen et al., 2022). High TSH, on the other hand, has been associated with high-grade tumors and clinic stage in DTC (Fan et al., 2024; Avram et al., 2022). As a result, while these tests are good at picking up benign conditions, they are not good at detecting cancers.

1.15.2 Core Tumor Biomarkers in Thyroid Cancer

1.15.2.1 Biomarkers in Differentiated Thyroid Cancer (DTC)

Thyroglobulin (Tg) is an essential marker for PTC and FVPTC and is also useful for follow-up of patients after total thyroidectomy and radioactive iodine (RAI) ablation (Giovanella et al., 2024; Lu et al., 2024). After therapy, elevated or rising Tg levels imply remaining or recurrent disease (Signore et al., 2023; Chou et al., 2022). Nevertheless, anti-thyroglobulin antibodies (TgAb) can affect Tg measurements; therefore, T3- and T4-based Tg assays will also require antibody testing for reliable calculation (Giovanella et al., 2023).

1.15.2.2 Biomarkers in Medullary Thyroid Cancer (MTC)

A special medullary thyroid carcinoma marker, calcitonin, is widely accepted for diagnosis and follow-up (Liu et al., 2024; Kiriakopoulos et al., 2022). Elevated calcitonin during and after therapy indicates persisting disease or relapse (Wang et al., 2024; Censi et al., 2023).

Carcinoembryonic antigen (CEA) is also helpful in monitoring disease, particularly when associated with calcitonin (Passos et al., 2021).

1.15.3 Novel and Inflammation-Related Biomarkers

1.15.3.1 Inflammatory Parameters

The prognostic importance of systemic inflammation markers in thyroid cancer is increasingly recognized (Cai et al., 2024; Huang et al., 2022). Elevated NLR has been associated with advanced stage, extrathyroidal invasion, and lower disease-free survival (Modica et al., 2023; Offi et al., 2021). Similarly, increased PLR is an indicator of aggressive behavior in PTC (Li et al., 2022; Martin et al., 2021). Higher levels of C-reactive protein (CRP) are associated with lymph node invasion and adverse prognoses (Marques et al., 2021; Offi et al., 2021).

1.15.3.2 Liquid Biopsy and Molecular Biomarkers

Liquid biopsy tools offer non-invasive approaches to analyze CTCs, ctDNA, and cfDNA (Allen et al., 2024). This real-time molecular tracking of tumor processes is achieved noninvasively. However, clinical use is still hindered by the high fraction of non-cancer cfDNA, which is also elevated in benign settings (Wang et al., 2024). While infrequent at early stages, CTCs are frequently detected at the case level in metastatic disease, paralleling tumor burden and disease prognosis (Lawrence et al., 2023; Nguyen et al., 2023). Moreover, cfDNA analysis can additionally detect tumor-specific mutations, including BRAFV600E, RAS, and TERT promoter mutations, which offer diagnostic and prognostic value (Wijewardene et al., 2025; Niedziela et al., 2024; Sato et al., 2021; Suh et al., 2021).

1.15.4 Serum Calcium and Parathyroid Hormone (PTH) Dynamics in Thyroid Cancer

It is essential to monitor the calcium and PTH levels, particularly after thyroidectomy (Chen et al., 2021; Bumber et al., 2020). Surgical injury to the parathyroid glands can lead to temporary or persistent hypocalcemia; as a result, intensive monitoring of the patient and replacement therapy are necessary (Maheshwari et al., 2024; Sępek et al., 2023). Ongoing hypocalcaemia might also

imply that of irreversible hypoparathyroidism, where calcium and vitamin D supplementation is needed (Staubitz-Vernazza et al., 2024; Casey et al., 2023; Khatiwada et al., 2021). Similarly, unexplained PTH levels increasing without simultaneous hypocalcemia may also be indicative of parathyroid carcinoma – a deadly form of cancer which is frequently misdiagnosed as thyroid cancer (Yabanoglu et al., 2024; Sawhney et al., 2022).

1.15.5 Vitamin D's Role in Thyroid Cancer Risk and Progression

Furthermore, Vitamin D insufficiency has recently been associated with a higher risk of thyroid cancer, as well as more aggressive histopathological features and a worse clinical course (Babić Leko et al., 2023; Abuduwaili et al., 2022; Palanca et al., 2022). It could promote cancer by immune modulation and chronic inflammation (Muñoz et al., 2022; Bikle et al., 2022). On the contrary, if these levels are sufficient, they could exert a protective effect; if this is a real clinical correlation, a potential therapeutic strategy remains to be addressed in future investigations (Dallavalasa et al., 2024).

1.15.6 Thyroglobulin in Long-Term Surveillance

Thyroglobulin remains the most widely used biomarker for long-term surveillance of patients with differentiated thyroid cancer (Giovanella et al., 2024; Li et al., 2022). Target Tg level is undetectable after thyroidectomy; any subsequent detectable rise indicates possible recurrence (Giovanella et al., 2023; Zahra et al., 2021). Interference caused by TgAb must be corrected in Tg measurements to interpret the levels correctly. The sensitivity of technological assays enhances the accuracy of recurrence detection (Dwivedi et al., 2023).

While regular thyroid function tests are essential for overall endocrine evaluation, they are not particularly helpful in diagnosing cancer. Tumor markers such as thyroglobulin (TG) and calcitonin (CT) are essential tools for diagnosing and monitoring thyroid cancer. Emerging biomarkers, such as inflammation indices and liquid biopsy markers, show potential to improve

early diagnosis, risk stratification, and personalized treatment. Nonetheless, further prospective studies are warranted to validate these new markers and to successfully integrate them into clinical protocols for better treatment of thyroid cancer.

1.16 TNM Staging of Thyroid Cancer (AJCC, 8th Edition)

Thyroid cancer is staged using the AJCC 8th Edition TNM system, which is based on the size of the primary tumor (T), involvement of regional lymph nodes (N), and presence of distant metastasis (M) (Lamartina et al., 2018). It is widely accepted for DTC (including PTC and FTC) and MTC only. This is not true for anaplastic thyroid cancer (ATC), which is classified as Stage IV by definition due to its aggressive nature (Tuttle et al., 2017).

Table 1.1: TNM Staging Criteria for Thyroid Cancer (AJCC 8th Edition)

Category	**Description**
Primary Tumor (T)	
TX	Primary tumor cannot be assessed.
T0	No evidence of primary tumor.
T1	Tumor ≤2 cm in the most significant dimension, limited to the thyroid.
T1a	Tumor ≤1 cm.
T1b	Tumor >1 cm but ≤2 cm.
T2	Tumor >2 cm but ≤4 cm, limited to the thyroid.
T3	Tumor >4 cm or with minimal extrathyroidal extension (e.g., into sternothyroid muscle or perithyroid soft tissues).
T3a	Tumor >4 cm but limited to the thyroid.
T3b	Tumor of any size with gross extrathyroidal extension into strap muscles (sternohyoid, sternothyroid, thyrohyoid, or omohyoid).
T4	Tumor with extensive extrathyroidal extension.

T4a	Tumor invades subcutaneous soft tissues, larynx, trachea, esophagus, or recurrent laryngeal nerve.
T4b	Tumor invades prevertebral fascia, mediastinal vessels, or encases the carotid artery.
Regional Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed.
N0	No evidence of regional lymph node metastasis.
N1	Regional lymph node metastasis.
N1a	Metastasis to Level VI or VII (pretracheal, paratracheal, or prelaryngeal/Delphian lymph nodes).
N1b	Metastasis to unilateral, bilateral, or contralateral cervical (Levels I–V) or retropharyngeal lymph nodes.
Distant Metastasis (M)	
M0	No distant metastasis.
M1	Distant metastasis present (e.g., to lungs, bones, liver, or brain).

Table 1.2: Staging of Differentiated Thyroid Cancer (PTC and FTC) (AJCC 8th Edition)

Age Group	Stage	T Category	N Category	M Category
<55 Years	I	Any T	Any N	M0
	II	Any T	Any N	M1
≥55 Years	I	T1–T2	N0/NX	M0
	II	T1–T2	N1	M0
	II	T3	Any N	M0
	III	T4a	Any N	M0
	IVA	T4b	Any N	M0

1.17 Grading Thyroid Cancer: Integrating Histological and Molecular Insights

Thyroid cancer grading is a crucial histological tool that assesses tumor differentiation, structural patterns, and cellular proliferation. While staging systems like the TNM assess tumor size, nodal involvement, and distant metastasis, grading primarily focuses on the tumor's intrinsic biology, which helps predict its clinical behavior and guides treatment planning.

1.17.1 WHO Classification of Thyroid Tumors (2022 Update)

The 2022 WHO classification system integrates histological details with molecular findings, providing a comprehensive framework for grading thyroid tumors. Including intermediate-grade (Grade 2) lesions and proliferation markers, such as the Ki-67 index, enhances diagnostic precision and supports personalized treatment planning. Molecular markers, such as BRAF V600E and TERT mutations, further assist in stratifying risk and identifying potential therapeutic targets.

The 2022 version of the World Health Organization (WHO) classification brings into focus a more refined approach by combining histological morphology with molecular changes (Jung et al., 2022; Juhlin et al., 2022):

- **Benign Lesions:** Comprise FA and its variants, which may be papillary or oncocytic.
- **Low-Risk Neoplasms:** This category includes the noninvasive follicular thyroid neoplasms with papillary-like nuclear features (NIFTP) and hyalinizing trabecular tumors, which are considered indolent.
- **Malignant Tumors:** Subdivided into:
    - **Well-differentiated carcinomas:** PTC and FTC, which are usually associated with a good prognosis.

- **High-Grade Carcinomas:** Consist of PDTC and ATC, which are well-known for aggressive behavior and poor prognosis.

Differentiated High-Grade Thyroid Carcinoma (DHGTC) is a significant new inclusion characterized by well-differentiated morphology that exhibits high-grade features, including marked mitotic activity and necrosis. This suggests a worse outcome.

1.17.2 Histological Grading According to Tumor Differentiation

Thyroid neoplasms are classified into four grades according to histological aspect and proliferative activity (Cracolici et al., 2023; La Rosa, 2023):

- **Grade 1: Well-Differentiated Carcinomas**
  - Have the same cellular structure as normal thyroid.
  - Primarily includes PTCs and FTCs.
  - Slow growing and has an excellent prognosis.
- **Grade 2: Moderately Differentiated Carcinomas**
  - There is a partial loss of normal architecture, with some "follicular"/ "glandular" patterns still visible.
  - Commonly occurs in aggressive PTC variants (tall cell, hobnail, columnar) and some FTCs.
  - Have moderate mitotic activity and necrosis, if present.
  - Ki-67 index: 3–10%, indicating moderate proliferation.
- **Grade 3: Poorly Differentiated Carcinomas (PDTC)**
  - Solid, island-forming, or trabecular in growth.
  - o">High mitotic index and high incidence of necrosis.
  - Associated with an increased chance of recurrence and worse survival.
  - Mitotische Tätigkeit: ≥3 per 2 mm²; Ki-67-Index: 10–30%.

- **Grade 4: Anaplastic Thyroid Carcinomas (ATC)**

    - o Made up of undifferentiated cells that do not resemble thyroid epithelium.
    - o Highly malignant with rapid progression.
    - o Mitotic count: ≥ 20 per 2 mm²; Ki-67 index: >30%.
    - o Usually presents with extensive necrosis and is clinically aggressive.

1.17.3 Specific Grading Criteria by Tumor Type

Regimen-specific grading systems have been developed for various tumor types, such as DHGTC, PDTC, and MTC, based on morphological patterns and mitotic activity, incorporating parameters like tumor necrosis and proliferation indices (Xu et al., 2023; Baloch et al., 2022).

- **DHGTC:**

    - o Morphologically high grade but with well-differentiated.
    - o Mitotic count increased: ≥5 per 2 mm² and/or necrosis.

- **PDTC:**

    - o Defined as lesions demonstrating insular, solid, or trabecular areas devoid of PTC-type nuclear features.
    - o Diagnostic features include:
        - Convoluted nuclei
        - Mitotic count ≥3 per 2 mm²
        - Tumor necrosis
    - o Ki-67 index: 10–30%.

- **Medullary Thyroid Carcinoma (MTC):**

    - o Grading is based on mitotic figure, Ki-67 index, and necrosis.
    - o High-grade MTC is defined by:
        - Mitotic count: ≥5 per 2 mm²

- Ki-67 index: ≥5%
- Tumor necrosis

1.17.4 Molecular Profiling of Tumors and Grading and Risk Assessment

Molecular profiling improves histological grading in terms of prognosis and therapy (Mukhtar et al., 2023; Harahap et al., 2023):

• BRAF V600E Mutation:

o Most commonly seen in the tall cell variant of PTC.

o With rising tumor aggressiveness and recurrence.

• TERT Promoter Mutations:

o More common in high-grade tumors.

o Associated with poor 5-year survival and poor clinical outcomes, it is also linked with reduced survival, increased metastatic ability, and worse outcomes.

• RAS Mutations:

o In follicular-patterned neoplasms such as FTC & NIFTP.

o Typically indicates sluggishness, but can be a driver of progression.

The 2022 WHO classification combines histology with molecular features to delineate clinically applicable correlations for thyroid lesion grading. The addition of intermediate-grade (Grade 2) lesions and proliferation markers, such as the Ki-67 index, increases diagnostic accuracy and enables a personalized approach to treatment. Molecular markers, such as BRAF V600E and TERT mutational status, also aid in risk stratification and the identification of potential therapeutic targets.

1.18 Risk Stratification Framework of the American Thyroid Association (ATA)

The American Thyroid Association (ATA) has generated a detailed risk-stratification system for DTC. The classification of patients into high-, intermediate-, and low-risk based on clinicopathological variables assists in prognosis predictions and tailors treatment management. Age is a significant prognostic factor, as increasing age is associated with higher recurrence rates and disease-specific mortality, which, in turn, affects the intensity of therapeutic management (Markantes et al., 2021).

1. Low-Risk Group

This group comprises patients at low risk of recurrence and with a good prognosis. Hallmark features include:

- Tumors limited to the thyroid without extrathyroidal extension, vascular invasion, or aggressive histologic subtypes.
- Papillary microcarcinomas (≤1 cm in diameter) limited to the thyroid gland.
- Follicular carcinomas that are surrounded by a capsule and have no vascular ingrowth.
- No such aggressive or high-risk genetic mutations as BRAF V600E

It is common for younger patients, especially those <55 years old, to be present in such a group, and these patients typically have better clinical outcomes with a low risk of recurrent disease (Maino et al., 2024).

2. Intermediate-Risk Group

Intermediate-risk patients have characteristics that confer a moderate risk of recurrence. These may include:

- Microscopic extrathyroidal extension into other structures.
- The tall cell, hobnail, and columnar cell types are histological variants of PTC for their aggressive nature.
- Restricted locoregional disease (in general, fewer than five cervical metastatic lymph nodes <3 cm).

- Vascular invasion with follicular or Hürthle cell carcinomas but no distant metastasis.
- Presence of one of the following positive molecular features: BRAF V600E mutation or TERT promoter mutation.

This population is more frequently found among patients aged 55 and older due to the relatively increased risk of recurrence (Moon et al., 2022).

3. High-Risk Group

Patients considered to be high risk are at a much higher risk of disease recurrence and cancer-specific death due to thyroid cancer. The defining criteria include:

- Macroscopic extrathyroidal extension (into trachea, esophagus, or the soft tissues).
- Presence of metastasis at first diagnosis.
- Advanced lymph node metastasis, particularly in nodes larger than 3 cm.
- Poorly differentiated carcinoma diagnosed/to be dedifferentiated into anaplastic carcinoma.
- High risk molecular profiles, specifically concomitant BRAF V600E/TERT promoter mutations or TP53 mutations.

Patients aged 55 and older in this group usually face worse outcomes and often need more aggressive and multimodal treatment approaches (Cavalheiro et al., 2021).

This system is one of the cornerstones in optimizing individual patient treatment and balancing disease control with treatment-associated risk. Its predictive accuracy is improved by adding age and molecular profiles, so that individuals at high risk, especially aged patients, receive the intensive care they need.

1.19 Targeted Gene Panel Sequencing via Next-Generation Sequencing in Oncology Research

Target gene panel sequencing, a subset of Next-Generation Sequencing (NGS), has become an essential tool for cancer research. This method selectively analyzes cancer-related genomic regions

of interest to achieve higher levels of deep sequencing and sensitivity. As a result, it helps identify low-frequency variants and uncover cancer pathogenesis, which can be utilized in precision cancer medicine (Satam et al., 2023; Willard et al., 2022).

Development and Implementation of Targeted Panels

Purposefully selecting cancer genes or genomic regions is expected to further characterize targeted panels, based on existing databases and the scientific literature. The goal of these panels is to cover clinically actionable alterations associated with diagnosis, prognosis, and therapeutic management (Pei et al., 2023; Zhong et al., 2021). Some facilities, such as Glasgow's Precision Oncology Laboratory, have developed tumor type-agnostic pan-cancer panels that cover a variety of tumor types, including solid and hematologic malignancies (Casolino et al., 2024). These panels are increasingly applied in clinical trials and are being evaluated for routine clinical applications.

Benefits of Targeted Panel Sequencing

There are some advantages of targeted sequencing in comparison to more comprehensive approaches, such as whole-genome (WGS) or whole-exome sequencing (WES):

- **Cost-efficient:** Data from specific regions comprise only a small portion of the reads, and thus have lesser costs, which is vital for applications that might be part of routine clinical assessments (Brlek et al., 2024).
- **Greater Sensitivity and Resolution:** Deep sequencing coverage (average 500x to 1000x) ensures that rare variants can be readily detected (Singh et al., 2022).
- **Simplified Interpretation:** Due to a smaller number of genes, the data is easy and less time-consuming to interpret (Damaraju et al., 2024).
- **Adaptability:** A panel can be customized or used commercially according to particular research or clinical needs (Pei et al., 2023).

Key Applications in Cancer Research

Cancer research as a focused application of targeted panel sequencing:

- **Molecular Tumor Characterization:** It identifies important oncogenic mutations for precision treatment (Akhoundova et al., 2022).

- **Minimal residual disease (MRD) monitoring:** This is the detection of low-frequency mutations in tissue or circulating DNA for use in evaluating recurrence or treatment response. This approach enables the identification of low-frequency mutations in tissue or circulating DNA, which is particularly important for testing recurrence or treatment response (Larribère et al., 2021).
- **Biomarker discovery:** Helps reveal the genetic modifications correlated with a drug sensitivity or resistance mechanism (Passaro et al., 2024).
- **Liquid biopsy:** It allows noninvasive ctDNA profiling for early detection, disease surveillance, and therapy assessment (De Luca et al., 2021).

Emerging Technological Innovations

Targeted panel sequencing has come of age with recent developments that have dramatically increased its power and applicability. Next-generation sequencers, such as the MiSeq i100 from Illumina, also offer more affordable, compact, and in-house NGS platforms for smaller laboratories (Zhong et al., 2021). Empiric targeted mutational profiling tools, such as the Ion AmpliSeq 50-Gene Cancer Hotspot Panel, facilitate sensitive and rapid detection of somatic mutations within oncogenes and tumor suppressors (Krishnamurthy et al., 2024).

Additional innovations include:

- **Ultra-Sensitive Detection Panels:** These improve the capability to identify ultra-rare variants in ctDNA, enabling earlier cancer detection (Semenkovich et al., 2023).
- **Dual-Modality Panels:** By integrating DNA and RNA analysis, these panels allow for the simultaneous detection of point mutations and gene fusions (Heydt et al., 2021).
- **AI-Enhanced Interpretation:** Machine learning tools are increasingly used to enhance the accuracy and efficiency of variant analysis and classification (Ozcelik et al., 2024).
- **Ultra-sensitive detection panels:** Increased sensitivity enables detecting ultra-rare variants in ctDNA and allows for earlier cancer diagnoses **(**Semenkovich et al., 2023**).**
- **Dual-Modality Panels:** Panels that combine both the analysis of DNA and RNA, enabling simultaneous measurement of both point mutations and gene fusion (Heydt et al., 2021).

- **AI-supported Interpretation:** In this work, machine learning tools are employed more and more to support the improved precision and effectiveness in variant analysis and classification (Ozcelik et al., 2024).

Challenges and Prospective Developments

Although targeted panel sequencing has its advantages, there are still several shortcomings:

- **Short Coverage:** Panels can only cover pre-determined mutations, and it is possible to miss rare or novel mutations that are not present in the design (Zalis et al., 2024).
- **Panel Design Constraints:** The diagnostic capability is closely related to the panelists and technicians who set the included targets (Yin et al., 2021).
- **Bioinformatics Overhead:** To accurately perform variant calling and annotation, a degree of computational infrastructure and technical resources is required (Singh et al., 2021).
- **Health Care Equity:** Wide usage must address the access of different communities to avoid disparities in cancer diagnosis and therapy (Dutta et al., 2023).

The evolving landscape of targeted sequencing will likely include the integration of multi-omic data tiers, the refinement of bioinformatic algorithms, and the development of personalized dynamic panels in line with emerging genomic knowledge.

The growing tool chest of options in the burgeoning field of cancer genomic approaches has led to the emergence of targeted panel sequencing as an indispensable tool in the balancing act among clinical relevance, cost, and depth of analysis. Despite its association with numerous diagnostic approaches such as tumor genotyping, liquid biopsies, and minimal residual disease (MRD) monitoring, it is the realm of precision oncology that reflects its continued deployment. Continued work on panel content, integration of novel technologies, and broad translation into research and clinical settings is essential to unlocking the full translational potential of this platform.

1.20 Ion AmpliSeq™ Cancer Hotspot Panel v2: An Advanced Targeted Sequencing Platform for Cancer Genomics

The Ion AmpliSeq™ Cancer Hotspot Panel v2 is a powerful targeted next-generation sequencing (NGS) assay designed to detect mutations in 50 predominant oncogenes and tumor suppressor genes commonly associated with human cancers (Lee et al., 2018). The optimized workflow for this panel focuses on PCR-based library preparation, SBS using the Ion Torrent technology, and automated variant analysis. Critically, the assay is compatible with low-input DNA (10 ng), making it well-suited to challenging clinical samples, such as formalin-fixed paraffin-embedded (FFPE) specimens. Consisting of 207 amplicons and targeting approximately 2,800 hotspot mutations reported in the COSMIC database (Messaoudi et al., 2022), the panel supports high-throughput profiling across solid tumors, including lung, breast, colorectal, ovarian, prostate, and melanoma.

Core Features and Technical Strengths

This panel targets hotspots across 50 genes, enabling the sensitive detection of clinically actionable mutations. It offers a high analytical sensitivity with a low limit of detection, i.e., down to 5% of variant allele frequency (VAF) for single-nucleotide variants (SNVs) and about 20% for small insertions and deletions (indels). The compatibility with degraded FFPE DNA even provides more promise for its use in routine diagnostics and in retrospective studies (Butler et al., 2016; Tafe et al., 2016).

Applications in Translational Cancer Research

The Ion AmpliSeq™ Cancer Hotspot Panel v2 has been successfully applied in many cancer genomics research studies. For example, in a project involving breast cancer patients from Saudi Arabia, DNA samples from peripheral blood were derived and sequenced with this panel, with hot-spot SMs detected in genes PIK3CA and TP53 occurring at a frequent basis, indicating the potential for this panel in the discovery of the mutation profiles that could be beneficial in personalized medicine (Messaoudi et al., 2022). The same was found for its use in non-small cell lung cancer (NSCLC) studies, where the transferability and high concordances of somatic mutations (including EGFR variants) were observed through ultra-deep sequencing of circulating cell-free DNA (cfDNA), supporting its potential for non-invasive cancer diagnostics and

longitudinal disease monitoring (Drejeriene et al., 2024). Moreover, a multisite assessment of the panel demonstrated flawless concordance with reference molecular tests, thereby confirming clinical reliability and diagnostic accuracy in everyday oncology practice (Casula et al., 2023).

Limitations and Considerations

Although it has many merits, the panel's primary weakness is a bias toward hotspot regions, which may lead to the omission of novel or rare mutations outside these regions. Furthermore, the platform has been developed specifically for Ion Torrent sequencers, which could limit its practical use in laboratories with non-NGS instruments (Bellevicine et al., 2016; Burghel et al., 2015).

The Ion AmpliSeq™ Cancer Hotspot Panel v2 provides a powerful solution with broad utility in research and clinical settings for rapid and sensitive identification of somatic mutations across multiple cancers. With the wide applicability of TMP, LB, and BD for tumor mutation profiling, liquid biopsy analysis, and biomarker development, TMPBoost becomes a promising tool for precision oncology. Its continued validation in multiple cancer types and integration into clinical genomics pipelines attest to its importance in cancer treatment and personalized medicine.

1.21 Purpose and Specific objective of the study

General objective of the study

The objective is to investigate the genotypic and biochemical profiles related to papillary thyroid carcinoma (PTC) in the Bangladeshi population. The objectives are to establish molecular biomarkers for early detection, assess their association with disease risk and tumor characteristics, and investigate their relationship with core biochemical measures.

Specific objectives

- To explore the molecular biomarker landscape for papillary thyroid cancer in the Bangladeshi cohort.
- To evaluate if the specified genetic variations are tumor stage or high/low-risk group-specific.

- To compare various biochemical blood parameters (TSH, Vitamin D, calcium, thyroglobulin, parathyroid hormone) between healthy individuals and those with diseases.

1.22 Significance of the study

There has been outstanding progress in the past decade in unraveling the molecules involved in thyroid carcinogenesis. Somatic mutations, as well as other molecular changes, have been identified as useful diagnostic and prognostic markers for thyroid cancer. Several genetic and epigenetic alterations, especially MAPK and PI3K–AKT activating mutations, lead to the development of thyroid cancers. However, the genetic factors related to thyroid cancer have not been extensively studied in the population of Bangladesh. To overcome this challenge, we propose to study, for the first time in Bangladesh, the molecular biomarker landscape of PTC by performing a population-based genome-wide association analysis. The biochemical profile related to thyroid functions in this group will also be investigated. Early detection of genetic predisposition using relevant genetic and biochemical markers, along with increasing knowledge in individuals carrying high-risk alleles, represents a potential prevention strategy for PTC. Furthermore, by examining the relationship between specific genetic changes and different biochemical blood data, new biomarkers and targeted therapeutic strategies for PTC patients can be explored.

1.23 Hypothesis of the Study

We assume that specific genetic changes, especially those that activate the MAPK and PI3K–AKT pathways, make individuals in the Bangladeshi population susceptible to developing PTC. Additionally, we suggest that such molecular biomarkers are associated with specific tumor features, such as tumor stage and risk group. They influence important blood biochemical parameters, including TSH, vitamin D, calcium, thyroglobulin, and parathyroid hormone. Discovering these associations would enhance our understanding of the molecular and biochemical factors underlying PTC in Bangladesh, thereby expanding the tools for more effective diagnostic and prognostic testing tailored to this population.

Chapter 2

Materials and Methods

2.1 Study Design

This research was conducted as a cross-sectional comparative study involving three distinct groups: 110 individuals diagnosed with papillary thyroid carcinoma (PTC), 100 patients with non-cancerous thyroid conditions, and 100 healthy controls. The study period spanned from September 2021 to June 2025. Diagnosis and staging of PTC cases were confirmed through histopathological examination and solid biopsy results following established diagnostic protocols and the World Health Organization (WHO) classification criteria (Jo et al., 2014).

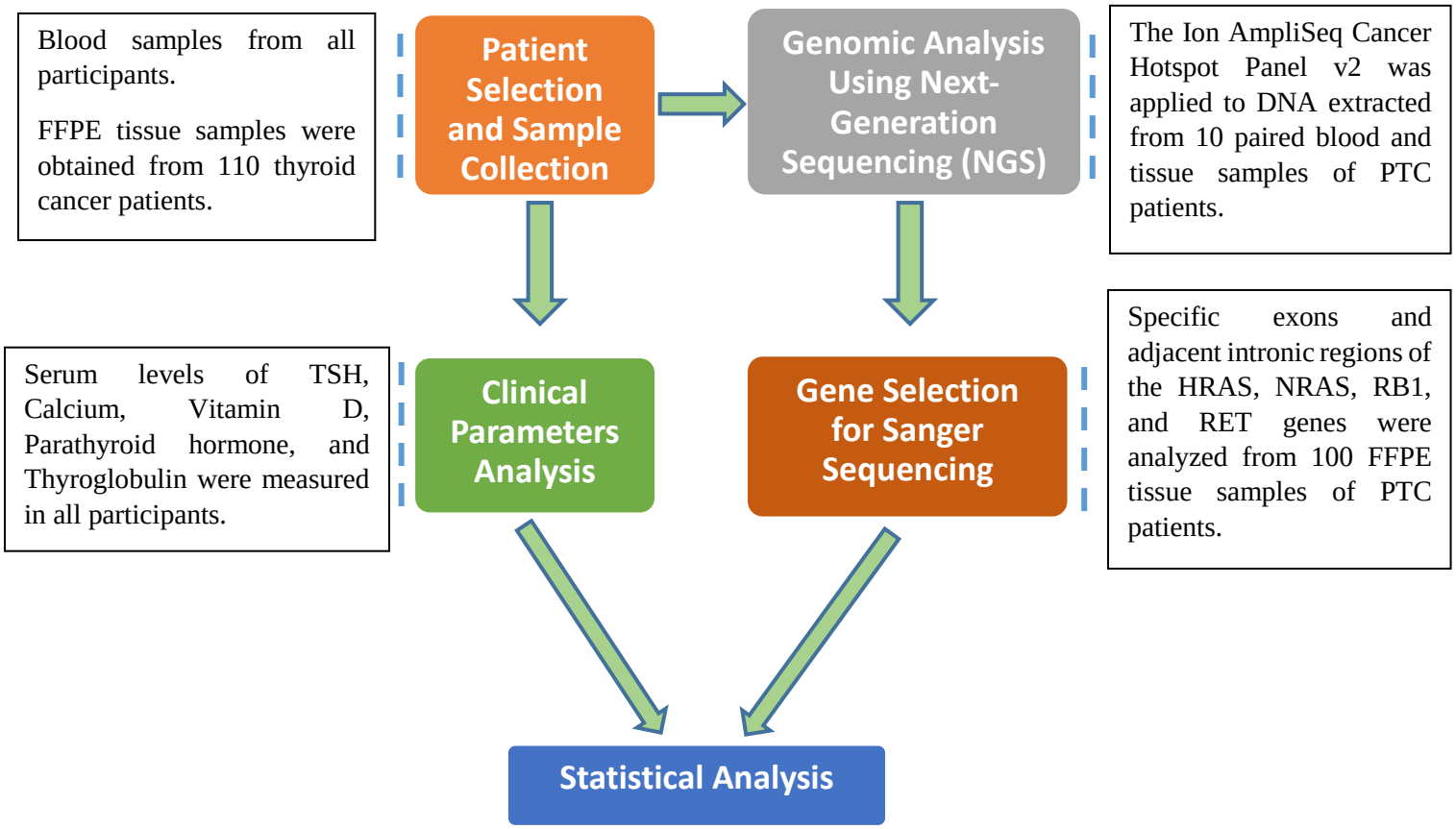


Figure 2.1: Schematic Representation of the Study Design.

2.2 Study Population

Participants were recruited from the National Institute of Nuclear Medicine and Allied Sciences, Bangladesh Medical University (BMU), based on comprehensive clinical and pathological

documentation. The age of patients with PTC ranged from 16 to 70 years. The healthy control group consisted of individuals with normal serum TSH levels and no history of chronic illnesses, including diabetes, cardiovascular disease, cancer, arthritis, or asthma. PTC cases were systematically classified according to established guidelines, including those of the World Health Organization (WHO), the American Joint Committee on Cancer (AJCC), and the American Thyroid Association (ATA). Classifications covered risk stratification, histopathological subtypes, TNM staging, and tumor grading (Baloch et al., 2022; Giuliano et al., 2022).

2.3 Eligibility Criteria for Study Participants

Participants were enrolled based on specific inclusion and exclusion criteria tailored to each group:

- **Papillary Thyroid Cancer (PTC) Patients**
  - *Inclusion Criteria*: Patients with a histologically confirmed diagnosis of PTC who had completed initial surgical treatment through total thyroidectomy.
  - *Exclusion Criteria*: Patients with a history of recurrent PTC or prior treatment with radioactive iodine (^{131}I) therapy.
- **Thyroid Patients Without Cancer**
  - *Inclusion Criteria*: Patients with elevated serum TSH levels (>5.00 ng/mL) and no evidence of malignancy.
  - *Exclusion Criteria*: Patients with any confirmed diagnosis of thyroid cancer.
- **Healthy Individuals**
  - *Inclusion Criteria*: Individuals with serum TSH levels within the normal reference range (0.30–5.00 ng/mL) and no history of chronic illnesses.
  - *Exclusion Criteria*: Individuals with chronic conditions such as diabetes, cardiovascular disease, cancer, arthritis, or asthma.

2.4 Questionnaire and Data Collection

Data were collected using a structured, pre-tested questionnaire designed to obtain comprehensive demographic and clinical information. Variables included age, gender, family history of thyroid

or other cancers, chronic illness history, and detailed smoking exposure. For patients with PTC, clinical features, including pathological tumor stage and other relevant tumor characteristics, were also systematically recorded.

2.5 Ethical Considerations and Approval

The study was conducted in accordance with the principles outlined in the Declaration of Helsinki and received ethical clearance from the Ethical Review Committee of the Faculty of Biological Sciences, University of Dhaka (Helsinki, 2013). All ethical protocols were strictly adhered to in accordance with international research guidelines. Each participant was fully informed of the study's objectives, and written consent was obtained, including permission to access relevant medical records. Participants were assured of their right to withdraw from the study at any time without penalty or impact on their medical care.

Confidentiality of all personal and medical information was rigorously maintained. Data were anonymized to protect individual identities and used exclusively for scientific research, handled in accordance with the highest standards of privacy and ethical compliance.

2.6 Collection and Storage of Blood Samples

After obtaining informed consent, approximately 6 mL of venous blood was collected from each participant by a trained professional using sterile, disposable syringes under strict aseptic conditions. Of the total blood volume, 3 mL was immediately transferred into an EDTA-containing tube (1.20 mg/mL) and stored at –20°C until DNA extraction. The remaining blood was transferred to a plain tube, kept chilled in an ice box, and transported to the laboratory. Upon arrival, the samples were centrifuged at 3,000 rpm for 15 minutes, and the separated serum was collected and stored at –20°C for future biochemical analysis.

2.7 Collection and Storage of FFPE Tissue Samples

Formalin-fixed, paraffin-embedded (FFPE) tissue samples were obtained from papillary thyroid cancer (PTC) patients following surgical excision and histopathological confirmation. Tissue specimens were fixed in 10% neutral-buffered formalin for 24–48 hours, processed, and embedded in paraffin blocks according to standard histological protocols. Each block was labelled with a unique identifier to ensure traceability while maintaining patient confidentiality.

FFPE tissue blocks were stored at room temperature in a dry, dark environment to preserve nucleic acid integrity until further molecular analysis. Thin sections (5–10 µm) were subsequently cut using a microtome and transferred to sterile, nuclease-free microcentrifuge tubes for downstream DNA extraction and mutation profiling. All procedures were performed in accordance with standard laboratory biosafety and biospecimen handling protocols.

2.8 Extraction and Quantification of DNA

2.8.1 Extraction of Genomic DNA from Whole Blood

Genomic DNA was extracted from peripheral whole blood using the FavorPrep™ Blood Genomic DNA Extraction HE Mini Kit (Favorgen Biotech Corp., Taiwan) according to the manufacturer's protocol. All reagents were prepared before extraction, and a dry/water bath was preheated to 60°C for the lysis step. To enhance DNA yield, the Elution Buffer was also pre-warmed to 60°C before the final elution.

Reagents Used

The reagents and components from the kit used for the extraction process were as follows:

- **Proteinase K** – For the enzymatic lysis of proteins
- **FABG Buffer** – Cell lysis and binding buffer
- **W1 Buffer** – First ethanol-containing wash buffer
- **Wash Buffer** – Second ethanol-containing buffer for membrane purification

- **Elution Buffer** – For eluting DNA from the column membrane
- **96–100% Ethanol** – Added to W1 and Wash Buffers before use
- **Phosphate-Buffered Saline (PBS)** – Used to adjust volume if the sample was <300 µL

DNA Extraction Protocol

1. **Sample Preparation**

   A total of 300 µL of whole blood was transferred into a 1.5 mL microcentrifuge tube. If the volume was insufficient, it was adjusted to 300 µL using phosphate-buffered saline (PBS).

2. **Cell Lysis**

   To initiate cell lysis, 30 µL of Proteinase K and 330 µL of FABG Buffer were added to the sample. The mixture was pulse-vortexed and incubated at 60°C for 15 minutes, with occasional vortexing.

3. **Precipitation and Binding**

   Following lysis, the sample was briefly cooled, and 330 µL of ethanol (96–100%) was added and mixed thoroughly.

4. **Column Binding**

   The prepared mixture was transferred to a FABG HE Mini Column, placed in a collection tube, and centrifuged at 6,000 × g for 1 minute. The flow-through was discarded.

5. **Washing**
   - 500 µL of W1 buffer was added to the column and centrifuged at 12,000 × g for 1 minute.
   - 900 µL of Wash Buffer was then added and centrifuged at 12,000 × g for 1 minute.
   - An additional dry spin was performed at full speed for 2 minutes to eliminate residual ethanol.
6. **Elution**

   The column was transferred to a clean elution tube, and 30 µL of pre-warmed (60°C) elution buffer or nuclease-free water (pH 7.5–9.0) was applied directly to the membrane.

After a 5-minute incubation at room temperature, DNA was eluted by centrifugation at 12,000 ×g for 1 minute.

2.8.2 Extraction of Genomic DNA from FFPE Tissue

Genomic DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tissue sections using the QIAamp® DNA FFPE Tissue Kit (QIAGEN), following the manufacturer's protocol. The procedure was optimized to ensure efficient DNA recovery while minimizing degradation typically associated with formalin fixation, thereby preserving sample integrity.

Reagents and Materials Used

The reagents and components from the kit used for the extraction process were as follows:

- **Proteinase K-** for the enzymatic lysis of proteins
- **Buffer ATL** – Lysis buffer
- **Buffer AL** – Lysis buffer for DNA binding
- **Buffer AW1 and AW2** – Wash buffers
- **Buffer ATE** – DNA elution buffer
- **Xylene** – For paraffin removal
- **Ethanol (96–100%)**
- **QIAamp MinElute Columns and 2 ml Collection Tubes**
- **RNase A (optional, for RNA-free DNA)**
- **1.5 ml and 2 ml microcentrifuge tubes**

All centrifugation steps were conducted at room temperature (15–25°C) using a standard microcentrifuge. Additionally, all buffers were equilibrated to room temperature before use.

Extraction Protocol Overview

1. **Deparaffinization**

   Up to 8 sections (5–10 μm each) were cut from the FFPE tissue block. The sections were transferred into microcentrifuge tubes, and 1 ml of xylene was added. The tubes were

vortexed and then centrifuged at maximum speed for 2 minutes. The supernatant was carefully discarded.

2. **Ethanol Wash**

   The pellet was washed with 1 ml of 96–100% ethanol, then vortexed and centrifuged. The supernatant was carefully removed. Any residual ethanol was evaporated by incubating the sample at room temperature or up to 37°C for approximately 10 minutes.

3. **Tissue Lysis**

   The pellet was resuspended in 180 µl of Buffer ATL and 20 µl of Proteinase K. The mixture was incubated at 56°C for 1 hour to ensure complete tissue digestion.

4. **Crosslink Reversal**

   The samples were incubated at 90°C for 1 hour to partially reverse formalin-induced crosslinking.

5. **RNA Removal (Optional)**

   For RNA-free preparations, 2 µl of RNase A (100 mg/mL) was added and incubated at room temperature for 2 minutes.

6. **DNA Binding**

   A total of 200 µl of Buffer AL and 200 µl of ethanol were added to the lysate and thoroughly mixed. The entire mixture was transferred to a QIAamp MinElute column and centrifuged.

7. **Washing Steps**

   The column was sequentially washed with 500 µl of Buffer AW1 and 500 µl of Buffer AW2, and centrifugation was performed after each wash. A final dry spin was performed to remove residual ethanol.

8. **Elution**

   DNA was eluted in 20–100 µl of Buffer ATE by incubating the buffer on the column for 1 minute, followed by centrifugation at maximum speed to collect the purified DNA.

9. **Storage of Extracted DNA**
   Eluted DNA was stored at –20°C for long-term preservation. For immediate downstream applications, short-term storage at 4°C was sufficient.

2.9 Quantification of DNA

After extraction, the concentration and purity of genomic DNA obtained from peripheral blood and FFPE tissue samples were assessed using a NanoDrop 1000 spectrophotometer. This quantification step ensured that nucleic acids were of sufficient quality and quantity for downstream molecular applications, including PCR, genotyping, and sequencing.

Measurement Procedure

1. **Sample Preparation**
   For DNA quantification, 1–2 µL of each sample was directly used for measurement. Dilution was not required unless the concentration of the sample exceeded the linear detection range of the instrument.

2. **Spectrophotometric Assessment**
   DNA concentration was determined by measuring absorbance at 260 nm (A260), the wavelength at which nucleic acids exhibit maximum absorption. Purity was assessed using the A260/A280 ratio:
   - A ratio of ~1.8 was considered indicative of pure DNA**.**
   - Ratios below 1.7 suggest possible l protein contamination.
   - Ratios above 2.0 indicated potential RNA contamination.
3. **Data Interpretation**:
   - DNA yield was reported in ng/µL.

- Samples with acceptable purity (A260/A280 ratio between 1.7 and 2.0) and concentrations greater than 20 ng/µL were considered suitable for downstream molecular applications.

4. **Storage**

   Quantified DNA samples were aliquoted and stored at –20°C for short-term use or at –80°C for long-term storage to prevent degradation.

5. **Quality Control**

   To assess DNA integrity, selected samples were further validated using 1% agarose gel electrophoresis. Intact, high-molecular-weight DNA appeared as a distinct, sharp band, whereas smeared patterns indicated partial degradation. Samples showing degradation were excluded from further analysis.

2.10 Targeted Sequencing Using Ion AmpliSeq™ Cancer Hotspot Panel v2

2.10.1 DNA Extraction and Quantification

Genomic DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tumor tissues using the QIAamp DNA FFPE Tissue Kit (Qiagen), following the manufacturer's protocol. The concentration and purity of the extracted DNA were measured using a Qubit Fluorometer (Thermo Fisher Scientific) and a NanoDrop spectrophotometer (Thermo Fisher Scientific). Only DNA samples with A260/280 ratios between 1.8 and 2.0 and sufficient concentrations (10–30 ng/µL) were selected for downstream analyses.

2.10.2 Library Preparation

Library construction was performed using the Ion AmpliSeq™ Library Kit 2.0 in conjunction with the Ion AmpliSeq™ Cancer Hotspot Panel v2 (Thermo Fisher Scientific), which targets mutational hotspots in 50 cancer-related genes. For each sample, 10–30 ng of genomic DNA was subjected to multiplex PCR amplification with two primer pools. The resulting amplicons were partially

digested with FuPa reagent, followed by adapter ligation with the Ion Xpress™ Barcode Adapters and the Ion P1 Adapter. The ligated libraries were subsequently purified using AMPure XP magnetic beads (Beckman Coulter).

2.10.3 Library Quantification

Purified libraries were quantified using the Ion Library Quantitation Kit on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Libraries were then diluted to the concentrations recommended for template preparation on the Ion Chef instrument.

2.10.4 Template Preparation and Chip Loading

Template preparation was performed using the Ion Chef™ System, which automates emulsion PCR, enrichment, and chip loading. Libraries were clonally amplified onto Ion Sphere particles (ISPs) by emulsion polymerase chain reaction (emulsion PCR). Template-positive ISPs were enriched and subsequently loaded onto Ion 530™ chips according to the sequencing run scale.

2.10.5 Sequencing

Sequencing was performed on the Ion S5 or Ion S5 XL System using Ion S5 sequencing reagents. The system uses semiconductor-based sequencing technology with 200-bp read-length chemistry. Sequencing runs were monitored using the embedded software in the Ion Torrent instrument interface.

2.10.6 Base Calling, Alignment, and Variant Calling

Base calling, read trimming, and quality filtering were performed using Ion Torrent Suite Software (version 5.x). High-quality reads were aligned to the human reference genome (hg19/GRCh37) with the Torrent Mapping Alignment Program (TMAP). Variant calling was conducted using the Torrent Variant Caller (TVC) plugin with default somatic settings, optimized to detect single-

nucleotide variants (SNVs) and small insertions or deletions (indels). Variants were filtered with a minimum coverage depth of 100× and a variant allele frequency (VAF) cutoff of 5%.

2.10.7 Variant Annotation and Data Analysis

Variant annotation and interpretation were conducted using Ion Reporter™ Software, a cloud-based platform. Annotated variants were filtered based on quality metrics, population database frequencies, and predicted functional impact. Additional annotation and cross-validation were performed with ANNOVAR and VEP (Variant Effect Predictor), incorporating public databases such as COSMIC, ClinVar, and dbSNP. Only non-synonymous variants, splice-site alterations, and known pathogenic mutations were retained for downstream analysis.

2.10.8 Data Visualization and Manual Review

Filtered variants were manually reviewed using **Integrative Genomics Viewer (IGV)** to ensure reliability**.** Read depth, strand bias, and sequence context at each variant site were inspected, and variants were confirmed only when supported by reads from both strands with consistent base quality.

2.10.9 Variant Interpretation and Clinical Significance

Variant interpretation followed the joint guidelines of the Association for Molecular Pathology (AMP), the American Society of Clinical Oncology (ASCO), and the College of American Pathologists (CAP) for classifying somatic variants (Li et al., 2017). Variants were categorized based on their predicted pathogenicity, therapeutic actionability, and association with cancer phenotypes.

The potential functional impact of nonsynonymous single-nucleotide variants was evaluated using in silico prediction tools such as SIFT (Sorting Intolerant From Tolerant; Vaser et al., 2016) and PolyPhen-2 (Polymorphism Phenotyping v2; Adzhubei et al., 2013). These algorithms assess the consequences of amino acid substitutions by analyzing evolutionary conservation, protein

structural context, and physicochemical properties, thereby indicating the likelihood that a variant will be deleterious or functionally neutral.

Additionally, curated databases such as OncoKB and MyCancerGenome were consulted to annotate the clinical relevance of identified mutations, including their prognostic, diagnostic, and therapeutic implications (Gazola et al., 2024).

2.11 PCR Primer Design

The design of high-quality primers is a critical step in achieving specific and efficient amplification in polymerase chain reaction (PCR)-based molecular assays. In this study, a rigorous, multi-step strategy was employed to design and validate primers targeting regions of interest with high fidelity.

Initially, the desired amplicon size was defined, typically ranging between approximately 400 bp and 1000 bp, to ensure optimal amplification efficiency while minimizing nonspecific binding and secondary structure formation. Using NCBI Primer-BLAST (Jian Ye et al., 2012), primer pairs were generated with the search parameters tailored for the *Homo sapiens* genome and the "Nucleotide collection (nr)" database. To streamline the selection process, output was restricted to ~20 candidate primer sets.

Thermodynamic constraints were applied, including melting temperatures (Tm) of ~55°C to 65°C, with ≤3°C difference between forward and reverse primers to ensure synchronized annealing. GC content and primer lengths were evaluated to enhance binding stability under standard PCR conditions. From the list of generated candidates, the most suitable primer pair was selected based on matched Tm values and GC content, while ensuring low levels of self-complementarity and 3'-end complementarity (each ≤ 3.00), which minimizes the risk of primer-dimer formation and nonspecific amplification. The specificity of the selected primer pair was validated using the UCSC In-Silico PCR tool (Kent et al., 2002), which confirmed the expected product size and the target locus. Chromosomal localization information derived from next-generation sequencing data was also used to refine primer positioning.

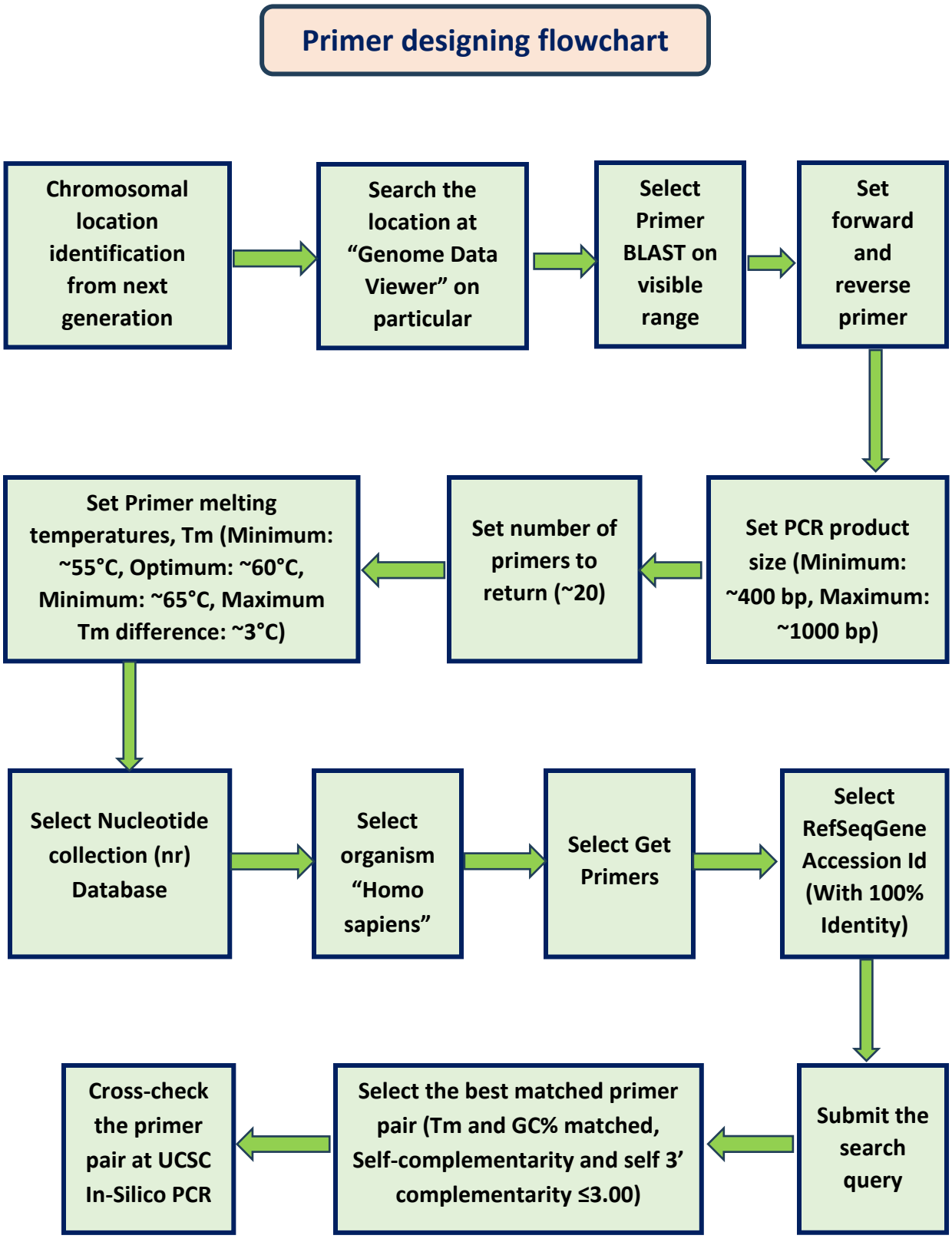


Figure 2.2: Schematic Representation of Primer Designing Strategy.

The precise genomic coordinates of each target region were verified using the NCBI Genome Data Viewer (https://www.ncbi.nlm.nih.gov/gdv?org=homo-sapiens&group=hominoidea)**,** referencing the appropriate human genome assembly GRCh38 (hg38). This step ensured that the primers flanked the intended loci without overlapping repetitive or highly polymorphic sequences, thereby enhancing the reliability of downstream amplification.

Collectively, this meticulous and computationally guided primer design pipeline enabled the selection of high-specificity, thermodynamically stable primer pairs, providing a robust foundation for reproducible and accurate molecular analyses.

Table 2.1: PCR Mixture Composition for Targeted Gene Amplification

Name of the Components	Volume (µL)
DreamTaq Master mix	15.0
NF water	6.0
Forward primer	2.5
Reverse primer	2.5
DMSO (10%)	1.0
Genomic DNA	3.0
Total	**30.0**

2.12 Target-Specific PCR Primer Information for Sanger Sequencing

2.12.1 Primer Details

Details of the primer sequences, product size, Tm, primer size, and chromosomal location are summarized in Table 2.2.

Table 2.2: PCR Primers Details of the Targeted Genes for Sanger Sequencing

Gene Name	Gene sequence accession number	Sequence Name	Sequence	Primer location	Primer length (bp)	Tm (°C)	Product Length (bp)
HRAS	NG_007666.1	HRAS F	TAAGAGCAAGTGGGGGCGAG	5976-5995	20	61.9	767
		HRAS R	CAAACACACACAGGAAGCCC	6723-6742	20	59.61	
NRAS	NG_007572.1	NRAS F	AATCTGTAGCCTCCTGGCTG	7600-7619	20	59.16	745
		NRAS R	GGTTCCAAGTCATTCCCAGT	8325-8344	20	57.41	
RET	NG_007489.1	RET F	CTGGGACACTCTGGGGAAAG	46059-46078	20	59.67	896
		RET R	CTATCCCTCTCGCACACACC	46935-46954	20	59.9	
RB1	NG_009009.1	RB1 F	GGGGGAAAGAAAAGAGTGGT	160795-160814	20	57.32	684
		RB1 R	GCCTTAGGTAGACGGATCA	161460-161478	19	55.61	

2.12.2 Optimized PCR Parameters for Target Gene Amplification

Polymerase Chain Reaction (PCR) was performed to amplify specific genomic regions before Sanger sequencing. Each reaction was carried out in a total volume of 30 µL, optimised according to the primer melting temperature (Tm), amplicon length, and GC content of the target region. The PCR mixture contained 15.0 µL of DreamTaq Master Mix (2X), 6.0 µL of nuclease-free water, 2.5 µL each of forward and reverse primers (typically 10 µM), 1.0 µL of DMSO (10%), and 3.0 µL of genomic DNA template (~50–100 ng) (Table 2.3). The thermal cycling conditions were adjusted for each primer pair based on the annealing temperature to ensure specific and efficient amplification.

Table 2.3: PCR Conditions for the Selected Genes

Gene Name	Denaturation Temperature with Time Duration	Cyclic Denaturation, Annealing, and Elongation Temperature with Time Duration	Number of Cycles	Final Elongation Temperature with Time Duration	Cooling Temperature with Time Duration
HRAS	95°C for 5 minutes	95°C for 60 Seconds 66°C for 60 Seconds 72°C 90 Seconds	40	72°C for 5 Minutes	4°C for ∞
NRAS	95°C for 5 minutes	95°C for 60 Seconds 62°C for 60 Seconds 72°C 90 Seconds	40	72°C for 5 Minutes	4°C for ∞
RET	95°C for 5 minutes	95°C for 60 Seconds 59°C for 60 Seconds 72°C 90 Seconds	40	72°C for 5 Minutes	4°C for ∞
RB1	95°C for 5 minutes	95°C for 60 Seconds 56°C for 60 Seconds 72°C 90 Seconds	40	72°C for 5 Minutes	4°C for ∞

Thermal cycling conditions typically consisted of an initial denaturation at 94–95°C for 2–5 minutes, followed by 30–35 cycles of denaturation (94–95°C for 30 seconds), annealing (Primer-specific, generally 55–62°C), and extension (72°C for 30–60 seconds, depending on amplicon size). A final elongation step was performed at 72°C for 5–10 minutes (Table 2.3). Amplification products were assessed by agarose gel electrophoresis (1.5%) to confirm expected size and quality before sequencing.

2.12.3 PCR Reagents

- DreamTaq Green PCR master mix (Promega, USA)
- Primers (New England Biolabs, USA)
- Agarose (Sigma Chemical Co., USA)
- 100 bp DNA ladder (Promega, USA)

2.13 Evaluation of PCR Amplification

The success and specificity of PCR amplification were evaluated by gel electrophoresis. An aliquot of 4 µL of each PCR product was loaded onto a 2% agarose gel Table 2.4) and separated under standard electrophoretic conditions. The expected amplicon sizes were verified by comparing the migration patterns against a 100 bp DNA ladder. Visualization of DNA bands was performed using Midori Green DNA Stain under UV illumination, and gel images were captured and archived for documentation and further analysis.

Table 2.4: Composition of 2% Agarose Gel (for 50 mL, 17-well casting tray)

Component	Amount
Agarose	1.0 g
Distilled Water	49.0 mL
50X TAE Buffer	1.0 mL
Midori Green DNA Stain	4.0 µL

Midori Green (https://www.nippongenetics.eu/en/products/electrophoresis-dna-rna/nucleic-acid-stains/midori-green-advance/) was incorporated directly into the molten agarose solution before casting the gel, allowing for direct visualization of DNA during UV exposure without the need for post-staining.

Reagent	Preparation Details
50X TAE Buffer	To prepare 1 L of 50X TAE buffer, 242 g of Tris base was dissolved in ~750 mL of deionized water using a magnetic stirrer. Subsequently, 57.1 mL of glacial acetic acid and 100 mL of 0.5 M EDTA were added. The solution was adjusted to **pH 8.3** and brought to the final volume with deionized water.
2.5% Agarose Gel	For higher-resolution separation, **2.5 g of agarose** (Invitrogen, Cat. No. 16500500) was dissolved in **100 mL of 1X TAE buffer** by heating in a microwave oven for ~2 minutes. The gel solution was cooled slightly, stained with **Midori Green**, and poured into a tray with a comb to form wells.
Midori Green Staining	**4.0 µL of Midori Green DNA stain** was added per 40 mL of agarose solution before casting the gel. No additional post-staining was required. Gels were imaged using a standard UV transilluminator.

2.14 Sanger Sequencing and Mutation Analysis

Mutation analysis of the target gene was performed using Sanger sequencing. Initially, 25 µL of the PCR product was purified using the EXOSAP PCR purification kit. Subsequently, 2.0 µL of the purified product was used for the sequencing reaction with the BigDye Terminator Sequencing Kit according to the manufacturer's protocol. Sequencing reaction products were further purified before capillary electrophoresis on the ABI 3500 Genetic Analyzer**.**

2.15 Analysis of Sequencing Data for Mutation Screening

Sequencing data were analysed using Geneious Prime Software (geneious.com). As only a one-month free trial version was available, data analysis was completed within the trial period.

2.15.1 Reference Sequence Selection and Sequence Alignment

The mutation analysis involved several key steps. First, consensus sequences were generated from the sample reads. Next, reference sequences were selected for alignment. Two approaches were used to obtain the reference:

1. Retrieval of the target gene reference sequence from the GenBank database (Clark et al., 2026)**,** and
2. In-silico PCR results generated using the NCBI Primer-BLAST and the UCSC In-Silico PCR tool.

Both approaches yielded identical reference sequences, confirming their accuracy. The in-silico PCR method provided the added advantage of including flanking regions on both ends of the exon. To further ensure reliability, exon sequences from Ensembl (Yates et al., 2020) and GenBank were aligned with the in-silico PCR results.

Following reference sequence selection, sample sequences were aligned and compared using BLAST, which confirmed a 100% match with the *Homo sapiens* gene, indicating high-quality, contamination-free sequencing data.

2.15.2 Quality Assessment and End Trimming

The sequencing read quality was assessed in Geneious Prime, which revealed low-quality base calls at the terminal regions (~40–50 nucleotides). These regions were removed using the software's default trimming parameters to improve downstream analysis.

2.16 Biochemical Assay

2.16.1 Vitamin D

Assay Methodology

Serum 25-hydroxyvitamin D [25(OH)D] levels were quantified using the ADVIA Centaur Vitamin D Total assay (Siemens Healthineers) on the ADVIA Centaur XP immunoassay analyzer, following the manufacturer's protocols (Siemens Document ID: DXDCM-09017) (www.siemens-healthineers.com).

Sample Preparation

The collected blood samples were allowed to clot at room temperature and centrifuged at 3,000 rpm for 15 minutes. The separated serum was aliquoted into labeled microtubes and stored at –20°C until analysis.

Principle and Procedure

The assay is based on a competitive chemiluminescent immunoassay that measures total 25(OH)D (D_2 and D_3). Endogenous vitamin D competes with a fluorescein-labeled analog for binding to a specific binding protein. The resulting complexes are captured by magnetic particles coated with anti-fluorescein antibodies, and a signal is generated using an acridinium-labeled anti-vitamin D antibody. The intensity of emitted light is inversely proportional to vitamin D concentration.

Reagents

- ADVIA Centaur Vitamin D Total Lite Reagent
- Solid Phase Magnetic Latex Particles
- Pretreatment Buffer (Reagents A, B, C)
- Calibrators and Quality Control (two levels)

Measurement and Calibration

Each assay was performed with 20 µL of serum. Relative light units (RLUs) were quantified against a calibration curve. Samples with concentrations exceeding 150 ng/mL were retested following a 1:2 dilution.

Reference Intervals

- Deficient: <20 ng/mL
- Insufficient: 20–29 ng/mL
- Sufficient: 30–100 ng/mL
- Toxic: >100 ng/mL

2.16.2 Parathyroid Hormone (PTH)

Assay Methodology

Serum intact PTH was measured using the ADVIA Centaur® Intact PTH assay (Siemens) on the ADVIA Centaur XP/XPT systems by a two-site sandwich chemiluminescent immunoassay (www.siemens-healthineers.com).

Procedure Overview

A 50 µL serum sample was incubated with an acridinium-labeled anti-PTH (N-terminal) antibody and streptavidin-coated magnetic particles pre-bound to a biotinylated anti-PTH (C-terminal) antibody. After washing, a chemiluminescent signal was generated and quantified.

Reagents

- Acridinium ester-labeled Lite Reagent
- Biotinylated Solid Phase Reagent
- Wash Buffer and Multi-Diluent
- Calibrators (two levels)

Performance Characteristics

- Measuring Range: 4.6–2000 pg/mL
- LoD: ≤6.0 pg/mL
- Precision: CV ≤8% for >20 pg/mL
- Reference Interval (Adults): 18.5–88.0 pg/mL

2.16.3 Thyroid-Stimulating Hormone (TSH)

Assay Methodology

Thyroid-stimulating hormone (TSH) levels were assessed using the Atellica® IM TSH 3-Ultra (TSH3UL) immunoassay on the ADVIA Centaur XP system. This third-generation chemiluminescent sandwich assay provides high sensitivity for evaluating thyroid function (www.siemens-healthineers.com).

Procedure

Each assay used 75 µL of serum. The reaction employed FITC-labeled and acridinium-labeled anti-TSH antibodies in combination with magnetic particles. Chemiluminescent signal intensity was directly proportional to the concentration of TSH.

Reagents

- Anti-TSH FITC-labeled and acridinium-labeled antibodies
- Magnetic solid phase particles
- Calibrators and quality controls

Measurement Range

- 0.008–150.000 µIU/mL
- Reference Interval (Adults): 0.55–4.78 µIU/mL

2.16.4 Thyroglobulin (Tg)

Assay Methodology

Serum Tg concentrations were measured using the IMMULITE® 2000 XPi Thyroglobulin assay (www.siemens-healthineers.com).

Assay Principle and Procedure

The assay employs a two-site chemiluminescent immunometric method. Serum (50 µL) was incubated with bead-bound anti-Tg antibodies along with an alkaline phosphatase-labeled polyclonal anti-Tg conjugate. The signal was generated by adding substrate and detected.

Reagents

- Polystyrene-coated beads with anti-Tg antibodies
- Alkaline phosphatase conjugate
- Chemiluminescent substrate
- Multi-level calibrators and controls

Measuring Range

0.2–300 ng/mL

Reference Interval (Adults): <55.00 ng/ml

2.16.5 Calcium

Assay Methodology

Calcium levels were measured using the Dimension® EXL™ with LM Integrated Chemistry System (Siemens), employing a photometric Arsenazo III dye-binding method (www.siemens-healthineers.com).

Procedure

Calcium concentration was determined by a colorimetric method in which calcium ions react with Arsenazo III to form a measurable color complex. The assay was fully automated and required a minimal sample volume (2–60 µL/test).

Reagents and Calibration

- Ready-to-use reagent cartridges
- Manufacturer-supplied calibrators
- Onboard reagent management

Measuring Range

Reference Interval (Adults): 8.04 - 10.30 mg/dL

Chapter 3

Results

3.1 Clinical Profile of the PTC Patient Cohort Selected for NGS Analysis

The next-generation sequencing (NGS) cohort included ten patients diagnosed with papillary thyroid carcinoma (PTC), representing clinical diversity in terms of age, sex, smoking history, family history, and tumor stage (Table 3.1). Females comprised 60% of the cohort, while males represented 40%. The patient age ranged from 18 to 65 years, with a median in the early 30s, consistent with the broad age distribution typically reported for PTC and its higher prevalence among young to middle-aged adults.

Table 3.1: Clinical Characteristics of Patients Selected for NGS Analysis

Patient ID	Gender	Age	Smoking Status	Family History of Cancer	Family History of Thyroid disease other than Thyroid tumor	TNM Staging
ATC 01	Female	19	No	Brain Tumor, Blood Cancer	No	pT2N1Mx
ATC 02	Female	29	No	No	Yes	pT1NxMx
ATC 03	Male	65	Yes	Thyroid carcinoma	Yes	pT2NxM1
ATC 04	Female	30	No	Thyroid carcinoma	Yes	pT1N1Mx
ATC 05	Male	54	Yes	No	Yes	pT2NoMx
ATC 06	Male	48	No	No	No	pT1NxMx
ATC 07	Male	35	Yes	Liver Cirrhosis	No	pT3NxMx
ATC 08	Female	18	No	No	Yes	pT3NxMx
ATC 09	Female	39	No	Cervical Cancer	No	pT1NxMx
ATC 10	Female	33	No	Esophageal Cancer	No	pT3NxMx

Smoking history was present in 30% of patients—all of whom were male—whereas none of the female participants reported smoking, reflecting broader gender-related lifestyle trends. More than half of the patients had a documented family history of malignancies, including thyroid carcinoma, brain tumors, hematologic cancers, cervical cancer, esophageal cancer, and liver cirrhosis,

suggesting a possible hereditary predisposition to cancer within the cohort. In addition, 50% of patients— predominantly females—reported a family history of non-neoplastic thyroid disorders, underscoring potential genetic or endocrine factors contributing to thyroid pathology.

Tumor staging, based on the TNM classification system, revealed heterogeneity in tumor burden and disease progression. Tumor size assessment (T stage) showed that 40% of tumors were classified as pT1, 30% as pT2, and 30% as pT3, indicating a spectrum ranging from small to locally advanced tumors. Lymph node status (N stage) was not evaluated in the majority of cases (Nx, 70%), while two patients demonstrated nodal metastasis (N1), and one patient was confirmed as node-negative (N0). Distant metastasis (M stage) was unassessed in the majority of cases (Mx, 90%), with one patient exhibiting confirmed distant metastatic spread (M1).

This cohort reflects the clinical and pathological heterogeneity characteristic of PTC, providing a robust foundation for exploring the correlation between genetic alterations, biochemical markers, and disease progression. The observed variability in staging and clinical features underscores the importance of integrating molecular profiling with phenotypic data to enhance understanding of PTC pathogenesis and identify potential diagnostic or prognostic biomarkers.

3.2 Panel-Based Next Generation Sequencing

3.2.1 Sequencing Quality Metrics for Paired Blood and Tissue Samples

High-quality sequencing performance was achieved across all ten paired blood and tissue samples analyzed using the Ion AmpliSeq™ Cancer Hotspot Panel v2, as evidenced by consistently robust sequencing metrics (Table 3.2). Genomic DNA concentrations exhibited expected variability between sample types. Blood-derived DNA yielded concentrations ranging from 31.4 to 81 ng/µL, whereas formalin-fixed paraffin-embedded (FFPE) tissue samples produced comparatively lower concentrations, ranging from 6.41 to 46.4 ng/µL. This reduction is consistent with the degradation and fragmentation commonly associated with FFPE-derived nucleic acids.

Table 3.2: Sequencing Read Statistics for Ten Paired Blood and Tissue Samples Analyzed Using the Ion AmpliSeq Cancer Hotspot Panel v2

Sample ID	DNA Conc. (ng/µL)	Library Conc. (ng/mL)	Coverage (X)	Mapped Reads	On Target (%)	Uniformity (%)
B001	63.3	6960	3415	720245	98.6	97.84
B002	39	6980	3460	731341	98.03	97.25
B003	37	7360	4144	881907	98.25	96.57
B004	50	6880	4918	1043369	98.57	97.41
B005	36.6	6760	4186	893011	97.88	97.18
B006	80	7640	4998	1062118	98.28	96.48
B007	81	7800	5746	1225117	98.86	95.62
B008	66	7720	5992	1280085	98.47	97.58
B009	71.5	7840	5687	1206298	97.64	97.42
B010	31.4	7360	6282	1334825	98.27	94.81
T001	34.47	6020	3542	846932	98.92	96.71
T002	24.66	6120	3645	699735	98.44	97.8
T003	16.87	10600	5157	1123715	98.16	97.95
T004	14.37	1092	3636	1159016	98.65	96.63
T005	6.41	7640	5528	1289359	97.54	96.73
T006	33.9	6360	3268	817389	97.98	97.41
T007	27.8	11240	4200	965889	98.85	94.16
T008	46.4	10520	4890	1067906	98.69	96.47
T009	7.25	6600	5021	1257126	98.19	97.58
T010	23.5	7720	5680	1296910	98.74	97.17
Mean	**39.57**	**7360.6**	**4669.75**	**1045115**	**98.35**	**96.84**

Library concentrations were uniformly high, indicating efficient library preparation. Blood-derived libraries ranged from 6,760 to 7,840 ng/mL, while tissue-derived libraries showed a broader range (6,020 to 11,240 ng/mL), likely due to variability in DNA input quality and

fragmentation. Sequencing depth, a critical determinant of mutation detection sensitivity, was reliably high across all samples. Blood samples achieved average coverage depths of 3415X to 6282X, while tissue samples ranged from 3268X to 5680X, ensuring sufficient depth for reliable variant calling.

The number of mapped reads per sample further validated sequencing quality, with blood samples yielding 720,245 to 1,334,825 reads and tissue samples 699,735 to 1,296,910 reads. Notably, on-target rates exceeded 97% across all samples, indicating high specificity in capturing the intended genomic regions. Coverage uniformity, a measure of read distribution across targeted regions, ranged from 94.81% to 97.84% in blood samples and 94.16% to 97.95% in tissue samples. These values fall well within acceptable ranges for targeted sequencing and demonstrate minimal amplification bias.

Collectively, these sequencing metrics confirm the reliability and efficiency of the Ion AmpliSeq™ Cancer Hotspot Panel v2 in generating high-quality data from both blood and FFPE tissue samples. This technical consistency provides a solid foundation for downstream mutation analysis, supporting the use of this platform in precision oncology studies.

3.2.2 Mutation Burden in Paired Blood and Tissue Samples

The total number of somatic mutations identified across the patient cohort demonstrated notable variability between blood and tissue samples, highlighting the underlying genetic heterogeneity and mutation burden in papillary thyroid carcinoma (Figure 3.1). In blood-derived DNA, the number of detected mutations ranged from 9 to 16 per patient, with some individuals exhibiting a markedly higher mutational load. Notably, patients ATC 01 and ATC 09 each harbored 16 mutations, representing the highest mutational burden within the blood sample group.

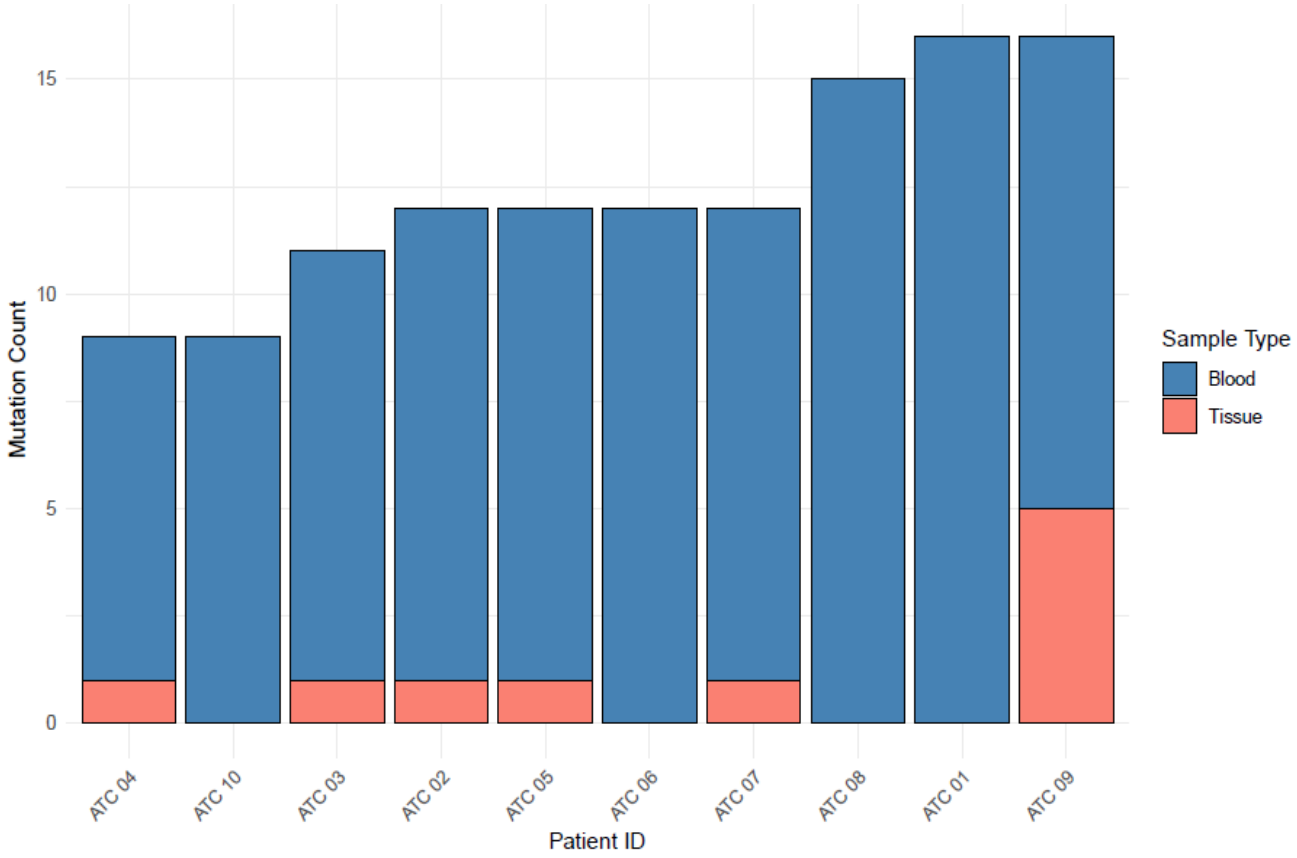


Figure 3.1: Mutation Frequency of Study Patients in Paired Blood and Tissue Samples.

In contrast, mutation frequencies in tissue samples were generally lower. Several patients, including ATC 01, ATC 06, ATC 08, and ATC 10, exhibited no detectable mutations in their tumor tissues. Others, such as ATC 02, ATC 03, ATC 04, and ATC 07, demonstrated 1 to 5 mutations. Interestingly, ATC 09, which showed one of the highest mutation loads in blood, also exhibited the most significant number of mutations (n = 5) in tissue, highlighting a potentially complex and patient-specific mutational landscape.

These observations suggest that, in most cases, the mutational burden in blood exceeds that of corresponding tissue, potentially reflecting differences in DNA quality, tumor heterogeneity, or the presence of circulating tumor-derived DNA. The observed interpatient variability further supports the need for conducting parallel analyses of both blood and tissue to characterize somatic alterations in PTC comprehensively.

3.2.3 Genomic Targets and Pathway Associations of Mutated Genes Identified in PTC Blood Samples

The panel of 27 unique genomic loci analyzed in this study comprises a mix of well-characterized oncogenes and tumor suppressor genes, each known to play pivotal roles in cancer biology, particularly thyroid tumorigenesis and associated signaling pathways (Table 3.3). These loci were selected based on their established clinical relevance, functional importance, and representation within key cancer-associated pathways. The selected mutations span both exonic and intronic regions, including functionally significant mutation hotspots, as well as regulatory elements in transcript splicing or expression.

The identified mutations were distributed across multiple chromosomes and mapped to precise genomic and transcript locations using reference transcript IDs (RefSeq). Genomic coordinates are reported according to the GRCh38/hg38 annotations. Many of the altered genes are integral components of key signaling networks, including MAPK, PI3K-Akt, RTK (receptor tyrosine kinase), Wnt/β-catenin, TGF-β, AMPK, Hedgehog, cell cycle control, and chromatin remodeling pathways.

For example, FGFR3, PDGFRA, KDR, RET, EGFR, HRAS, KIT, and MET are oncogenes involved in MAPK and RTK signaling, which are frequently associated with aberrant growth factor responses and uncontrolled proliferation. PIK3CA, FLT3, CSF1R, and SMO are drivers of PI3K-Akt and Hedgehog signaling pathways, often linked to enhanced survival and drug resistance. Classical tumor suppressor genes such as TP53, STK11, CDKN2A, SMAD4, FBXW7, and APC were mutated in these loci, affecting apoptosis, genomic stability, and cell cycle regulation. SMARCB1, a component of the SWI/SNF chromatin remodeling complex, was found to carry inactivating mutations that may lead to transcriptional dysregulation and tumor progression. The dataset also includes mutations in terminal exons (e.g., APC exon 17/17, SMARCB1 exon 9/9, PIK3CA exon 21/21, CSF1R exon 21/21), early exons (e.g., HRAS exon 2/5), and regulatory intronic regions (e.g., RET intron 15/19, SMAD4 intron 8/11, CDKN2A intron 1/3). Variations in these positions may have pathogenic significance by altering splicing patterns or disrupting gene expression.

Table 3.3: Gene Characteristics and Pathway Association of Identified Germline Mutations

Serial No.	Gene Name	Gene Type	Genomic Location	Chromo-somal Location	Transcript Location	Relevant Signaling Pathway	Exon/Intron number/Total number
01	FGFR3	Oncogene	Chr04:1806167	4p16.3	NM_000142.5	MAPK, RTK	Exon 14/18
02	PDGFRA	Oncogene	Chr04:54274888	4q12	NM_006206.6	RTK, PI3K-Akt	Exon 13/24
03	PDGFRA	Oncogene	chr04: 54285873	4q12	NM_006206.6	RTK, PI3K-Akt	Exon 19/24
04	TP53	Tumor suppressor	chr17:7676154	17p13.1	NM_000546.5	p53 signaling, Apoptosis	Exon 4/11
05	KDR	Oncogene	chr04:55114072	4q12	NM_002253.4	MAPK, PI3K-Akt	Exon 6/30
06	KDR	Oncogene	chr04: 55106807	4q12	NM_002253.4	MAPK, PI3K-Akt	Exon 11/30
07	KDR	Oncogene	chr04:55096379 - 55096380	4q12	NM_002253.4	MAPK, PI3K-Akt	Exon 18/29
08	APC	Tumor suppressor	chr05:112840073	5q22.2	NM_000038.6	Wnt/β-catenin pathway	Exon 17/17
09	RET	Oncogene	chr10:043118395	10q11.21	NM_020975.6	MAPK, RTK	Exon 13/20
10	RET	Oncogene	chr10:43120185	10q11.21	NM_020975.6	MAPK, RTK	Intron 15/19
11	PIK3CA	Oncogene	chr03:179199217	3q26.32	NM_006218.4	PI3K-Akt	Intron 2/20
12	PIK3CA	Oncogene	chr03:179234232	3q26.32	NM_006218.4	PI3K-Akt	Exon 21/21
13	FLT3	Oncogene	chr13:28036046	13q12.2	NM_004119.3	RTK, PI3K-Akt	Intron 10/23
14	FLT3	Oncogene	chr13:28028155	13q12.2	NM_004119.3	RTK, PI3K-Akt	Intron 16/23
15	CSF1R	Oncogene	chr05:150054033	5q32	3’ UTR: NM_001288705.3	RTK, PI3K-Akt	Exon 21/21
16	ERBB4	Oncogene	chr02:211947372	2q34	NM_005235.3	MAPK, RTK	Intron 3/27

17	SMARCB1	Oncogene	chr22:23834100	22q11.23	NM_003073.5	SWI/SNF complex, Chromatin remodeling	Exon 9/9
18	EGFR	Oncogene	chr07:55181370	7p11.2	NM_005228.5	MAPK, PI3K-Akt, RTK	Exon 20/28
19	STK11	Tumor suppressor	chr19:1220322	19p13.3	NM_000455.5	AMPK signaling	Exon 4/10
20	HRAS	Oncogene	chr11:534242	11p15.5	NM_001130442.3	MAPK, RTK	Exon 2/5
21	MET	Oncogene	chr07:116699618	7q31.2	NM_001127500.3	MAPK, PI3K-Akt, RTK	Exon 2/21
22	MET	Oncogene	chr07:116700208	7q31.2	NM_001127500.3	MAPK, PI3K-Akt, RTK	Exon 2/21
23	KIT	Oncogene	chr04:54727298	4q12	NM_000222.3	MAPK, PI3K-Akt, RTK	Exon 10/21
24	FBXW7	Tumor suppressor	chr04:152326126	4q31.3	NM_033632.3	Ubiquitination pathway	Exon 10/21
25	SMAD4	Tumor suppressor	chr18:51059974	18q21.2	NM_005359.6	TGF-β signaling	Intron 8/11
26	SMO	Oncogene	chr07:129205261	7q32.1	NM_005631.5	Hedgehog signaling	Exon 3/12
27	CDKN2A	Tumor suppressor	chr09:21971212	9p21.3	NM_001195132.2	Cell cycle, p53 signaling	Intron 1/3

This targeted panel comprises fifteen oncogenes and six tumor suppressor genes distributed across multiple chromosomal loci, including chromosomes 4, 5, 7, 9, 10, 11, 13, 17, 18, 19, and 22, which highlights the polygenic nature of papillary thyroid carcinoma. The integration of pathway annotation underscores the role of these genetic alterations in disrupting core cellular processes, thereby justifying their inclusion in downstream mutational profiling, pathway enrichment, and functional validation analyses.

3.2.4 Characterization and Functional Impact of Germline Variants Identified in Blood Samples

A total of 27 germline variants were identified from blood-derived DNA of papillary thyroid carcinoma (PTC) patients, encompassing synonymous, missense, intronic, and non-coding transcript variants (Table 3.4). These alterations were distributed across a panel of oncogenes and tumor suppressor genes commonly implicated in cancer biology. All variants were annotated using reference databases such as dbSNP and COSMIC, and their potential pathogenicity was evaluated using SIFT and PolyPhen-2 where applicable.

The majority of the detected variants were synonymous substitutions, which do not alter the encoded amino acid but may still influence splicing efficiency or mRNA stability. Notable examples include: FGFR3 (c.1953G>A; p.Thr651=) and PDGFRA (c.1701A>G; p.Pro567=), both detected in all individuals (10/10), suggesting a common polymorphic status. Additional synonymous changes were detected in APC, RET, PIK3CA, EGFR, HRAS, and MET, most of which were classified as benign based on existing annotations and computational predictions.

Missense variants, which introduce single amino acid substitutions and may alter protein function, were observed less frequently. Among these, TP53 (p.Arg72Pro) was detected in 90% of patients (9/10). This is a well-characterized polymorphism associated with cancer susceptibility in various populations. KDR (p.Gln472His) and SMO (p.Arg199Gln) were identified in 30% and 10% of individuals, respectively. While the KDR variant was predicted to be benign, the SMO variant yielded a PolyPhen score of 0.961, suggesting a potentially damaging effect and warranting further investigation. Rare missense mutations in MET (p.Asn375Ser) and KIT (p.Met541Leu) were also identified, though both were predicted to be functionally benign.

Several additional variants were identified within intronic regions or the 3′ untranslated region (UTR), including alterations in FLT3, KDR, SMAD4, ERBB4, CDKN2A, and CSF1R. Although these non-coding variants do not alter the protein sequence, they may affect splicing efficiency, regulatory element function, or transcript stability, particularly when located near exon-intron junctions. Most of these variants are currently cataloged as benign or of unknown clinical significance.

Table 3.4: Characteristics and Functional Impact Predictions of Germline Mutations Identified in Blood Samples

Serial No.	Gene Name	Nucleotide and amino acid change	Mutation Type	Annotation	%Mutation	SIFT Score	Poly-Phen Score	Pathology Prediction
01	FGFR3	c.1953G>A p.Thr651=	Synonymous	rs7688609	100	-	-	Unknown
02	PDGFRA	c.1701A>G (p.Pro567=)	Synonymous	rs1873778	100	-	-	Benign
03	PDGFRA	c.2472C>T p.Val824=	Synonymous	rs222823, COSM 22413	40	-	-	Benign
04	TP53	c.215G>C p.Arg72Pro	Missense	rs104252, COSM5346903	90	0.262	0.083	Benign
05	KDR	c.798+54G>A	Intronic variant	rs7692791	90	-	-	Unknown
06	KDR	c.1416A>T p.Gln472His	Missense	rs1870377 COSM149673	30	0.027	0.003	Benign
07	KDR	TGG>TGGG c.2615-37dup	Intronic variant, dupG	rs3214870	20	-	-	Unknown
08	APC	c.4479G>A (p.Thr1493=)	Synonymous	rs41115	90	-	-	Benign
09	RET	c.2307G>T p.Leu769=	Synonymous	rs1800861 COSM4418405	80	-	-	Benign
10	RET	c.2712C>G p.Ser904=	Synonymous	rs1800863, COSM 3751779	30	-	-	Benign
11	PIK3CA	c.352+40A>G	Intronic variant	rs3729674	80	-	-	Benign
12	PIK3CA	c.3075C>T p.Thr1025=	Synonymous	rs17849079 COSM21451	60	-	-	Benign
13	FLT3	c.1310-3T>C	Intronic variant	rs2491231 COSM3999060	70	-	-	Benign
14	FLT3	c.2053+23A>G	Intronic variant	rs75580865	10	-	-	Benign

15	CSF1R	c.*35_*36del insTC	Non Coding Transcript Variant, Downstream UTR variant	rs386693509	80	-	-	Unknown
16	ERBB4	c.421+58A>G	Intronic variant	rs839541	60	-	-	Benign
17	SMARCB1	c.1119-41G>A	Intronic variant	rs503061, COSM 1090	50	-	-	Benign
18	EGFR	c.2361G>A p.Gln787=	Synonymous	rs1050171	40	-	-	Benign
19	STK11	c.465-51T>C	Intronic variant	rs2075606	40	-	-	Benign
20	HRAS	c.81T>C p.His27=	Synonymous	rs12628, COSM 249860	20	-	-	Benign
21	MET	c.534C>T p.Ser178=	Synonymous	rs3577572, COSM 1579024	10	-	-	Benign
22	MET	c.1124A>G p.Asn375Ser	Missense	rs3391795, COSM 5020653	10	0.29	0.038	Benign
23	KIT	c.1621A>C p.Met541Leu	Missense	rs3822214, COSM 28026	10	0.804	0.012	Benign
24	FBXW7	c.1524A>G p.Gln508=	Synonymous	rs147462419	10	-	-	Unknown
25	SMAD4	c.955+58C>T	Intronic variant	rs948588	10	-	-	Benign
26	SMO	c.596G>A p.Arg199Gln	Missense	rs759466401, COSM6493918	10	0.388	0.961	Unknown
27	CDKN2A	c.151-4G>C	Intronic variant	rs529380972, COSM14254	10	-	-	Benign

Functional prediction scores, when available, reinforced the classification of most missense variants as tolerated (SIFT > 0.05) or benign (PolyPhen < 0.85). However, a small number of variants approached or exceeded pathogenicity thresholds, such as the SMO p.Arg199Gln, highlighting the need for further functional characterization or population-based assessments.

The frequency distribution of these germline variants revealed a combination of common polymorphisms and rare, low-frequency variants, reflecting both shared genetic backgrounds and possible individual-specific profiles. Collectively, these findings contribute to a broader understanding of germline genetic variation in patients with PTC and may offer insights into inherited predisposition. However, their functional and clinical significance remains to be established through tumor-normal comparisons and larger population-based studies.

3.2.5 Comparative Analysis of Allele Frequencies for Gene Variants Identified in Blood Samples across Global, Asian, and Study Populations

To investigate the population-level relevance of germline variants identified in blood-derived DNA from patients with papillary thyroid carcinoma (PTC), allele frequency data were compared across three cohorts: global populations, Asian populations, and the study cohort (n = 10) (Table 3.5). Reference and alternate allele frequencies were retrieved from public databases such as gnomAD and the 1000 Genomes Project, and cross-validated using Ensembl and dbSNP.

The analysis revealed substantial variability in allele distributions, reflecting both ancestry-specific polymorphism patterns and potential enrichment of specific variants within the study population.

High-Frequency Variants in the Study Cohort

Several alternate alleles were observed at 100% frequency (homozygous for the variant allele) in the patient cohort, despite their lower frequencies in global or Asian populations. Notably, FGFR3 rs7688609 (G>A) and PDGFRA rs1873778 (A>G) were present in all 10 patients (100%), yet showed global alternate allele frequencies of 98.7% and 98.4%, respectively, and were completely absent in the Asian reference population. This pattern suggests a potential founder effect or localized enrichment.

Other variants showed high enrichment in the tested group, including APC rs41115 (G>A) and CSF1R rs386693509 (TG>GA). The latter was absent in both the global and Asia populations but

present in 65% of the tested samples, indicating a possible novel or rare population-specific variant.

Table 3.5: Comparative Analysis of Allele Frequencies for Germline Mutations Identified in Blood Samples across Global, Asian, and Study Populations

Gene Name & rs ID	Allele Frequency (Global)		Allele Frequency (Asian)		Allele Frequency (Tested)	
	Reference Allele	Alternate Allele	Reference Allele	Alternate Allele	Reference Allele	Alternate Allele
FGFR3 rs7688609 (G>A)	0.0126	0.9874	0.0	1.0	0.0	1.0
PDGFRA rs1873778 (A>G)	0.0155	0.9845	0.002	0.998	0.0	1.0
PDGFRA rs2228230 (C>T)	0.8463	0.1537	0.8693	0.1307	0.65	0.35
TP53 rs1042522 (G>C)	0.2857	0.7143	0.4152	0.5848	0.4	0.6
KDR rs7692791 (C>T)	0.4609	0.5391	0.3654	0.6346	0.35	0.65
KDR rs1870377 (T>A)	0.7604	0.2396	0.527	0.473	0.7	0.3
KDR rs3214870 (TGG>TGGG)	0.7622	0.2378	0.583	0.417	0.8	0.2
APC rs41115 (G>A)	0.3872	0.6128	0.182	0.818	0.1	0.9
RET rs1800861 (G>T)	0.2272	0.7728	0.524	0.476	0.4	0.6
RET rs1800863 (C>G)	0.8145	0.1855	0.923	0.077	0.85	0.15
PIK3CA rs3729674 (A>G)	0.7795	0.2205	0.905	0.095	0.5	0.5
PIK3CA rs17849079 (C>T)	0.9922	0.0078	0.995	0.005	0.75	0.25
FLT3 rs2491231 (A>G)	0.2879	0.7121	0.26	0.74	0.45	0.55
FLT3 rs75580865 (T>C)	0.9397	0.0603	1.0	0.0	0.95	0.5
CSF1R rs386693509 (TG>GA)	0.0	0.0	0.0	0.0	0.35	0.65
ERBB4 rs839541 (T>C)	0.7443	0.2557	0.598	0.402	0.65	0.35

SMARCB1 rs5030613 (G>A)	0.8605	0.1395	0.94	0.06	0.75	0.25
EGFR rs1050171 (G>A)	0.4333	0.5667	0.804	0.196	0.75	0.25
STK11 rs2075606 (T>C)	0.7439	0.2561	0.5	0.5	0.7	0.3
HRAS rs12628 (A>G)	0.6568	0.3432	0.836	0.164	0.9	0.1
MET rs35775721 (C>T)	0.9506	0.0494	0.908	0.092	0.9	0.1
MET rs33917957 (A>G)	0.9817	0.0183	0.9319	0.0681	0.9	0.1
KIT rs3822214 (A>C)	0.9082	0.0918	0.9399	0.0601	0.9	0.1
FBXW7 rs147462419 (T>C)	1.0	0.0	1.0	0.0	0.9	0.1
SMAD4 rs948588 (C>T)	0.9291	0.0709	0.946	0.054	0.9	0.1
SMO rs759466401 (G>A)	1.0	0.0	1.0	0.0	0.9	0.1
CDKN2A rs529380972 (C>G)	0.9998	0.0002	1.0	0.0	0.9	0.1

Pathogenic or Functional Variants with Moderate Frequencies

Variants with established clinical or functional relevance were observed at moderate frequencies. For example, TP53 rs1042522 (G>C), a well-known polymorphism associated with cancer susceptibility, exhibited a global alternate allele frequency of 71.4%, an Asian frequency of 58.5%, and was present in 60% of the patient cohort. Similarly, KDR rs1870377 (T>A) and FLT3 rs2491231 (A>G) showed moderate frequencies across all three populations, suggesting that these are common variants that may modulate disease risk rather than act as primary causative factors.

Conserved Reference Alleles in Public Datasets but Present in the Cohort

Interestingly, several variants that are nearly absent in both global and Asian reference datasets were detected in the study cohort. For instance, SMO rs759466401 (G>A), SMAD4 rs948588 (C>T), and CDKN2A rs529380972 (C>G) were present in 10% of the tested individuals. Their

alternate allele frequencies in global and Asian populations were near zero, highlighting the possibility of low-penetrance or private variants specific to this population.

Well-Conserved Variants across All Populations

Specific variants, such as MET rs35775721 (C>T), KIT rs3822214 (A>C), and HRAS rs12628 (A>G), showed consistent allele frequencies across global, Asian, and tested populations. These likely represent ancestry-independent polymorphisms and may serve as internal controls in population genetic analyses.

This comparative analysis not only aids in classifying the significance of variants but also supports the development of a more accurate genetic risk profile for PTC in the South Asian population.

3.2.6 Genomic Targets and Pathway Associations of Mutated Genes Identified in PTC Tissue Samples

A total of eight genes with genomic alterations were identified in tissue-derived DNA from patients with papillary thyroid carcinoma (PTC) (Table 3.6). These genes include both oncogenes and tumor suppressors, many of which are recognized to play pivotal roles in key oncogenic signaling pathways. The identified variants span multiple exonic and intronic regions, with genomic coordinates mapped to the GRCh38/hg38 human genome build and annotated against RefSeq transcript references.

Among the oncogenes, several critical regulators of the MAPK, PI3K-Akt, and RTK (Receptor Tyrosine Kinase) pathways were identified: BRAF (chr7:140753336), a well-established driver in thyroid and other cancers, showed a variant in exon 15, a hotspot for activating mutations (e.g., V600E) associated with constitutive MAPK signaling. NRAS and HRAS, both members of the RAS family of GTPases, harbored variants in exon 3 and exon 2, respectively. These exons are known mutational hotspots that modulate downstream MAPK and PI3K-Akt pathway activity. MET, a receptor tyrosine kinase that mediates cellular growth and migration, showed a variant in exon 2, potentially affecting ligand binding or receptor activation.

The only tumor suppressor gene identified, RBI*, was found to be mutated in exon 20. RB1 is a critical regulator of the cell cycle and p53 pathways, and its inactivation is commonly associated with loss of cell cycle control and increased tumorigenic potential. This finding suggests a possible disruption of tumor-suppressive mechanisms in the analyzed tissue.

Table 3.6: Characterization of Genes and Functional Pathway Mapping from Tissue-Derived DNA

Serial No.	Gene Name	Gene Type	Genomic Location	Chromo-somal Location	Transcript Location	Relevant Signaling Pathway	Exon/Intron number/Total number
01	BRAF	Oncogene	chr07:140753336	7q34	NM_004333.6	MAPK, ERK, PI3K-AKT	Exon 15/18
02	NRAS	Oncogene	chr01:114713909	1p13.2	NM_002524.5	MAPK, RTK, PI3K-AKT	Exon 3/7
03	HRAS	Oncogene	chr11:534242	11p15.5	NM_001130442.3	MAPK, RTK	Exon 2/5
04	RB1	Tumor suppressor	chr13:48459766	13q14.2	NM_000321.3	Cell cycle regulation, p53, PI3K-AKT	Exon 20/27
05	MET	Oncogene	chr07:116699618	7q31.2	NM_001127500.3	MAPK, PI3K-Akt, RTK	Exon 2/21
06	KDR	Oncogene	chr04: 55096380	4q12	NM_002253.4	MAPK, PI3K-Akt	Intron 18/29
07	FLT3	Oncogene	chr13:28036046	13q12.2	NM_004119.3	RTK, PI3K-Akt	Intron 10/23
08	CSF1R	Oncogene	chr05:150054033 - 150054034	5q32	NM_001288705.3	RTK, PI3K-Akt	Exon 21/21

Variants were also detected in KDR (VEGFR2) and FLT3, both receptor tyrosine kinases involved in angiogenesis and hematopoietic signaling. The KDR variant was located in intron 18, and the FLT3 variant in intron 10, raising the possibility of non-coding regulatory effects or splicing alterations. CSF1R, a receptor involved in macrophage proliferation and immune signaling, exhibited a variant in exon 21, which may influence receptor function or downstream signaling cascades.

Collectively, the altered genes identified in tumor tissues are functionally linked to key cancer-related pathways, including MAPK, PI3K-Akt, RTK, cell cycle, and p53 signaling pathways. These pathways are well-established in thyroid cancer pathogenesis and may serve as potential therapeutic targets or biomarkers for tumor characterization. The distribution of mutations across both coding and non-coding regions underscores the importance of integrating structural and regulatory genomic data to understand the molecular basis of tumor behavior in PTC comprehensively.

3.2.7 Characterization and Functional Impact of Somatic Variants Identified in Tissue Samples

A total of eight somatic variants were identified in tumor tissue DNA derived from patients with papillary thyroid carcinoma (Table 3.7). These included missense, synonymous, intronic, and non-coding transcript variants, affecting both oncogenes and tumor suppressor genes that are known to play critical roles in thyroid and general oncogenic pathways. All variants were annotated using dbSNP and COSMIC, and their potential functional relevance was assessed using in silico prediction tools such as SIFT and PolyPhen-2.

The most clinically relevant alteration was observed in the BRAF gene (c.1799T>A; p.Val600Glu), detected in 3 out of 10 patients (30%). This mutation, known as BRAF V600E, is a well-established oncogenic driver in thyroid carcinoma, resulting in the constitutive activation of the MAPK/ERK pathway. Functional prediction scores confirmed its high pathogenic potential (SIFT: 0.001; PolyPhen-2: 0.971), supporting its critical role in tumor development and progression.

Additional pathogenic missense variants included: NRAS c.181C>A (p.Gln61Lys), found in 1 patient (10%), is another canonical hotspot mutation that dysregulates MAPK and PI3K-Akt signaling. It exhibited strong functional impact predictions (SIFT: 0.009; PolyPhen-2: 0.948). RB1 c.2039T>C (p.Ile680Thr), also seen in 1 patient (10%), may compromise cell cycle regulation and contribute to loss of tumor suppressor function (SIFT: 0.001; PolyPhen-2: 0.967).

Synonymous mutations, although silent at the protein level, were detected in HRAS (p.His27=) and MET (p.Ser178=). These variants are predicted to be benign, but may still influence splicing efficiency or mRNA stability under specific contexts.

Table 3.7: Characteristics and Functional Impact Prediction of Somatic Mutations in Tissue Samples

Serial No.	Gene Name	Nucleotide and amino acid change	Mutation Type	Annotation	%Mu-tation	SIFT Score	Poly Phen Score	Pathology Prediction
01	BRAF	c.1799T>A p.Val600Glu	Missense	rs113488022 COSM476	30	0.001	0.971	Pathogenic
02	NRAS	c.181C>A p.Gln61Lys	Missense	rs121913254, COSM580	10	0.009	0.948	Pathogenic
03	HRAS	c.81T>C p.His27=	Synonymous	rs12628, COSM 249860	10	-	-	Benign
04	RB1	c.2039T>C p.Ile680Thr	Missense	rs1949384455	10	0.001	0.967	Pathogenic
05	MET	c.534C>T p.Ser178=	Synonymous	rs35775721, COSM 1579024	10	-	-	Benign
06	KDR	TGG>TGGG c.2615-37dup	Intronic variant, dupG	rs3214870	10	-	-	Unknown
07	FLT3	c.1310-3T>C	Intronic variant	rs2491231 COSM3999060	10	-	-	Benign
08	CSF1R	c.*35_*36delinsTC	Non-Coding Transcript Variant, Downstream UTR variant	rs386693509	10	-	-	Unknown

Variants located in non-coding regions included: KDR c.2615-37dup (dupG) in intron 18, classified as an intronic duplication, and FLT3 c.1310-3T>C, a splice region variant in intron 10. While these variants are generally considered benign or of uncertain significance, they may affect

transcriptional regulation or alternative splicing. Additionally, the CSF1R (c.35_36delinsTC) variant, located within the 3′ untranslated region (3′ UTR), represents a downstream non-coding transcript variant. While its pathogenic potential remains unclear, such variants may influence post-transcriptional regulatory mechanisms.

Collectively, the somatic mutation landscape of the tissue samples comprises a combination of high-impact driver mutations (e.g., BRAF V600E, NRAS Q61K, RB1 I680T) alongside low-frequency or likely benign alterations. The functional annotation supports the involvement of key oncogenic pathways, including MAPK, RTK, PI3K-Akt, and cell cycle signaling, in the pathogenesis of PTC. These findings highlight both the heterogeneity of somatic mutations in thyroid tumors and the utility of targeted sequencing for identifying clinically actionable genetic events.

3.3 Selection of Candidate Genes for Downstream Sanger Sequencing

3.3.1 Details of Targeted Gene and Their Functional Pathways

Based on NGS results from paired tissue samples and data mining from the cBioPortal (https://www.cbioportal.org/) for Cancer Genomics, four genes—HRAS, NRAS, RET, and RB1—were selected for further genomic analysis (Cerami et al., 2012). These genes were prioritized due to their known oncogenic or tumor-suppressive roles in thyroid carcinogenesis, recurrent mutation profiles in thyroid and other cancers, and functional enrichment in cancer-critical signaling networks. Specific exons, along with adjacent intronic regions, were targeted to capture potential coding mutations, splicing alterations, and regulatory variants (Table 3.8).

Each of the selected genes contributes to well-established cancer signaling pathways such as RAS/MAPK, PI3K/AKT, JAK/STAT, and cell cycle regulation:

- HRAS, a classic oncogene within the RAS family, was targeted at exon 2 (nucleotides 6176–6339, 164 nts), which contains known activating mutations that result in constitutive downstream signaling via the MAPK and PI3K/AKT cascades.

- NRAS was examined at intron 2 and exon 3, covering nucleotides 7917–8095 (179 nts). Exon 3, particularly codon 61, is a known mutational hotspot in thyroid tumors and other solid cancers. The inclusion of intronic flanking sequences increases the likelihood of identifying splice-altering variants or cryptic regulatory elements.

Table 3.8: Characteristics of Selected Genes and Their Associated Signaling Pathways

Serial no.	Gene name	Gene type	Functional pathway	Intronic, Exonic number	Exonic position (exon length)
01.	HRAS	Oncogene	RAS/MAPK and PI3K/AKT	Exon 2	6176-6339 (164 nts)
02.	NRAS	Oncogene	RAS/MAPK and PI3K/AKT	Intron 2, Exon 3	7917-8095 (179 nts)
03.	RET	Oncogene	RAS/MAPK, PI3K/AKT and JAK/STAT	Intron 13, Exon 13	46305-46412 (108 nts)
04.	RB1	Tumor suppressor	Cell cycle regulation	Exon 20	160942-161087 (146 nts)

- RET, a proto-oncogene encoding a receptor tyrosine kinase, was analyzed at intron 13 and exon 13 (nucleotides 46305–46412, 108 nts). RET mutations are characteristic of specific PTC subtypes, and this gene integrates multiple pathways, including MAPK, PI3K/AKT, and JAK/STAT, making it a critical molecular node in thyroid cancer pathogenesis.
- RB1, a key tumor suppressor gene regulating the G1/S cell cycle checkpoint, was investigated at exon 20 (nucleotides 160942–161087, 146 nts). Mutations in this exon can impair the function of the RB1 protein, leading to the loss of cell cycle control and contributing to tumor progression.

These targeted loci were selected to enable sensitive detection of somatic mutations with potential pathogenic or prognostic relevance in PTC. Their inclusion reflects an integrative strategy combining experimental evidence from NGS with curated cancer mutation databases to enhance the biological interpretation of patient-specific genomic alterations.

3.3.2 Analysis of Gene Interaction Network

A gene-gene interaction network overview using GeneMANIA (http://genemania.org), which illustrates the protein interactions and co-expression relationships between candidate genes, is shown in Figure 1A (Franz et al., 2018). The resultant networks reflect the strength and connectivity of the links between the chosen candidates: HRAS, NRAS, RET, and RB1.

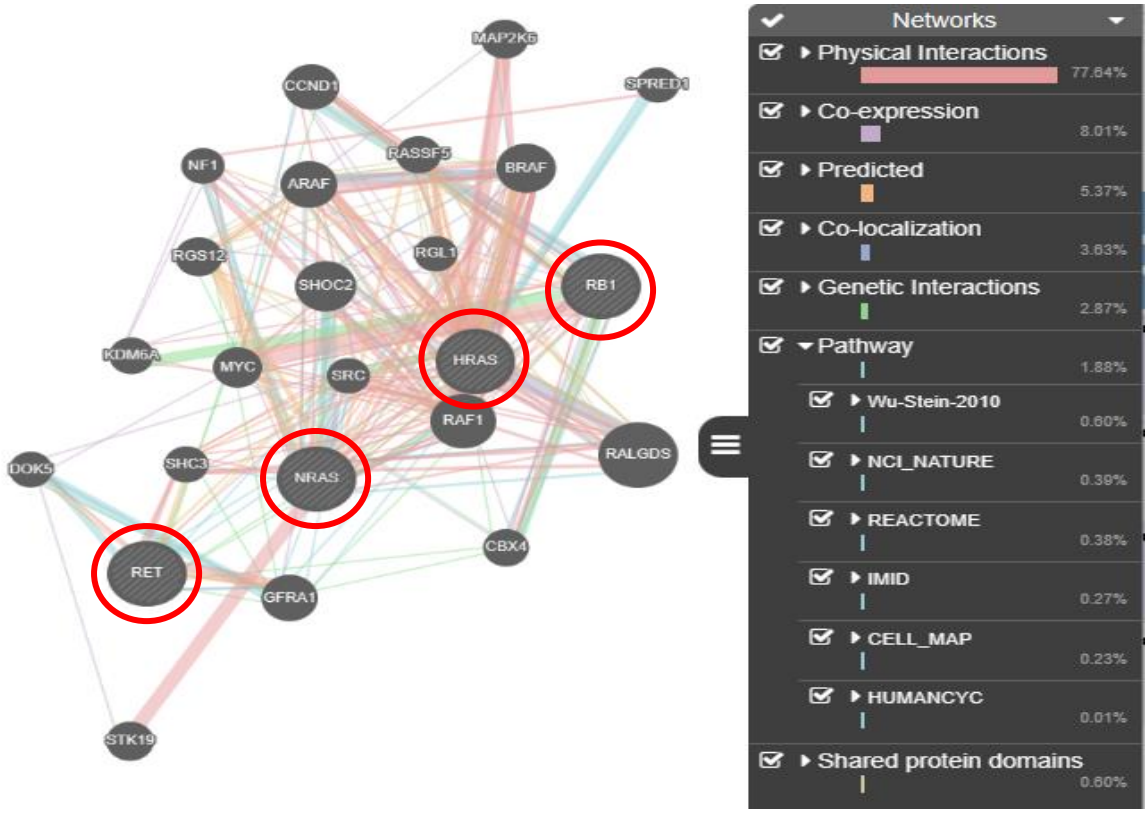


Figure 3.2: Interaction Network on Selected Oncogenes and Tumor Suppressor Genes.

At the center of the network stands HRAS, a hub, as it is highly interconnected, indicating a high level of integration with other molecules through the sharing of regulatory mechanisms and physical interactions. NRAS also presents a tight interconnectivity profile, including strong co-expression and functional links (the second case), placing the gene near HRAS in the network structure. RET, more downstream in the signaling network, remains in a functional context through several direct and indirect interactions, notably through physical and pathway-based connections. Through intergenic and domain-level interactions, RB1 interacts with several regulatory genes, substantiating its participation in the larger interaction networks.

This network map helps reveal the highly significant nature of these genes, which was subsequently proved, but also for showing them within a framework of indirect interaction that suggests, or better represents, the co-regulatory or co-functional relevance of these genes throughout thyroid cancer pathogenesis. Based on their network neighbors and connectivities, the arguments for their consideration for other mutational analyses in this study are justified.

3.4 Sanger Sequencing of Selected Target Genes

3.4.1 Baseline Characteristics of Study Subjects for Sanger Sequencing

For Sanger sequencing analysis, specific exonic and intronic regions of genes were selected in a cohort of 100 PTC patients using DNA from formalin-fixed paraffin-embedded (FFPE) tumor tissue samples. The age, sex distribution, clinical presentation, and tumor features were heterogeneous within the study cohort. The average age at diagnosis was 35.27 ± 1.167 years, and the average age at PTC onset was 33.47 ± 1.146 years. Regarding age, 20% of patients were 25 years or younger, 52% were between 26 and 39 years old, and 28% were 40 years or older (Table 3.9).

The female-to-male ratio for this cohort was 3:1, as expected given the female predilection in PTC incidence. Smoking history was present in fifteen patients, and the remaining 85 were non-smokers. The grading of the tumors revealed that the majority of patients (98%) had well-differentiated (G1) tumors, while only two patients exhibited grade 2 histology.

Based on the maximum dimension, the size of the tumor varied: 29 cases had a tumor with the maximum dimension less than 2 cm, 38 with a size of 2-3 cm, and 33 with a size more than 3 cm. Histopathological classification showed that 92% of patients had the classical subtype of PTC, and 8% had the follicular variant of PTC (FVPTC).

According to the TNM staging, 42 patients had small (T1) and non-invasive PTC, and 22 had medium-sized (T2) invasive ones. Extra thyroidal extension or larger bulk (T3) was considered as extensive disease in 36, and 14 presented with lymph node metastasis. Risk stratification indicated

that most patients (91%) were in the intermediate-risk group, seven were in the high-risk group, and only two were in the low-risk group as per the ATA criteria.

Table 3.9: Overview of Clinical and Demographic Parameters of the Sanger Sequencing Cohort (n = 100)

Variables		Value in number (n)
Age		
	≤25	20
	26-39	52
	≥40	28
	Mean± SEM	35.27±1.167
PTC Onset Age	Mean± SEM	33.47±1.146
Gender		
	Male	26
	Female	74
Smoking Status		
	Yes	15
	No	85
Tumor Grade		
	G1	98
	G2	2
Tumor Size (Greatest Dimension) in cm		
	<2	29
	2-3	38
	>3	33
Histopathological Classification		
	Classical	92
	Follicular Variant of Papillary Thyroid Carcinoma (FVPTC)	8
TNM Staging		
	Small, localized PTC (T1)	42
	Moderate-sized PTC (T2) without Lymph Node Metastasis	22

	Advanced PTC with Extra Thyroidal Extension or Larger Tumor Size (T3)	22
	PTC with Lymph Node Metastasis	14
Risk group		
	Low Risk	2
	Intermediate Risk	91
	High Risk	7
Family History of Thyroid Disease other than Thyroid Tumor		
	Yes	16
	No	84
Family History of Thyroid Tumor		
	Yes	19
	No	81
Family History of Cancer other than Thyroid		
	Yes	29
	No	71

The results are reported as numbers.

As for the family history, 16 patients had a history of non-tumorous thyroid disease in first-degree relatives and 19 patients had a family history of thyroid tumors. Furthermore, 29 patients had a history of cancer elsewhere in the family, suggesting a genetic component in some part of the cohort.

3.4.2 Assessment of PCR Amplification for Targeted Genes Used in Sanger Sequencing

PCR amplification was used for validation and generating specific amplicons in the selected genomic regions of RB1, NRAS, RET, and HRAS genes before Sanger sequencing. To verify the

expected product sizes and amplification specificity, the products were run on 2% agarose gels (Midori Green staining), which are shown in Figure 3.3.

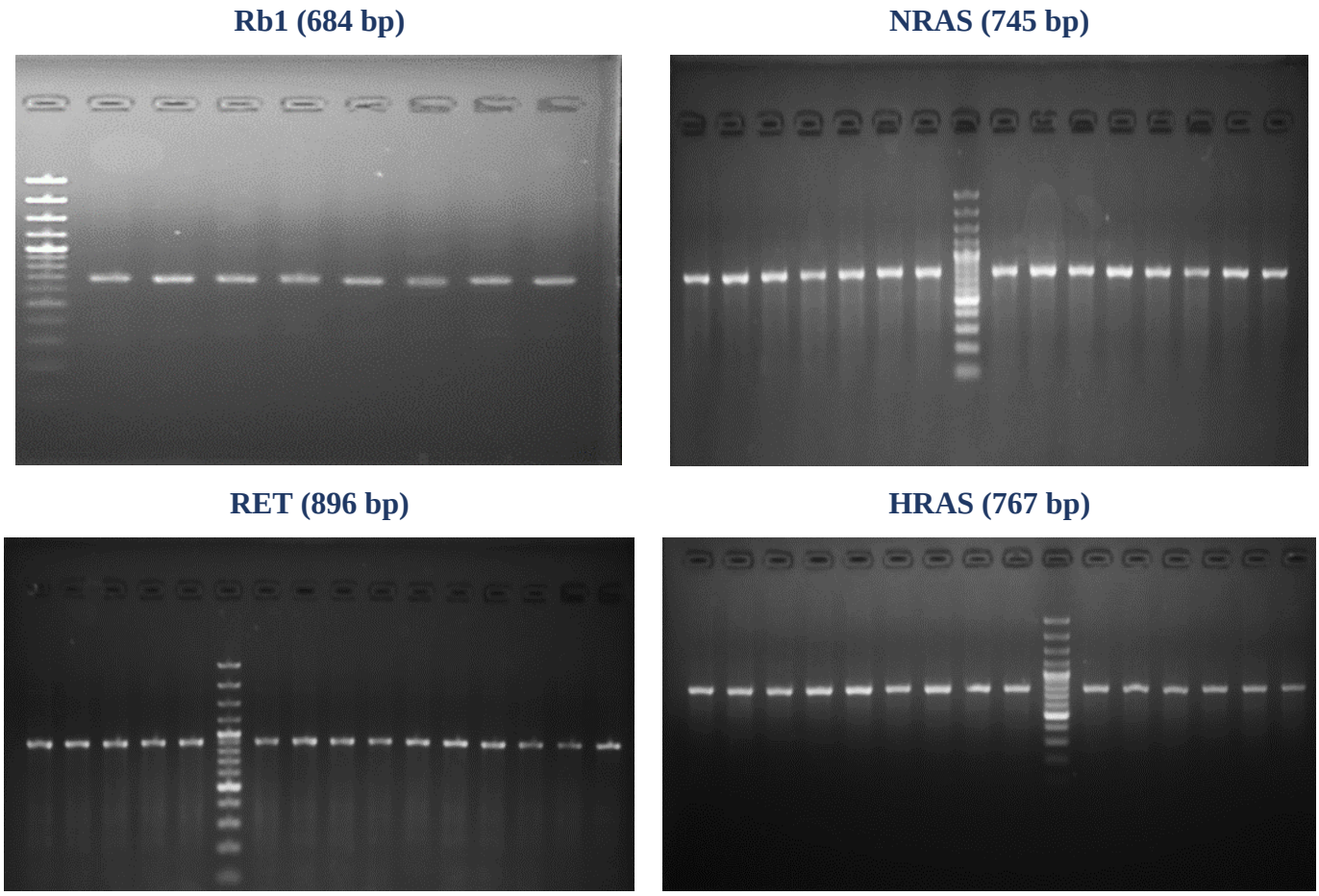


Figure 3.3: Representative PCR Amplification of Selected Genes on 2% Agarose Gel.

A GeneRuler™ 100 bp Plus DNA Ladder (Thermo Scientific, USA) was used as the molecular weight marker.

Figure 3.3 demonstrates that specific and obvious amplicons corresponding to the expected fragment lengths were effectively produced from the genomic DNA extracted from the FFPE tissue samples. The approximate band sizes were 684 bp for RB1, 745 bp for NRAS, 896 bp for RET, and 767 bp for HRAS. All primers designed specifically for individual genes successfully

produced a reliable amplification pattern in the tested cultivars, indicating the specificity and the efficiency of the PCR conditions established for each target.

A 100 bp Plus DNA Ladder (GeneRuler™, Thermo Scientific, USA) was used as a reference marker for size determination of the PCR products. The distinct banding with the absence of non-specific amplification or primer-dimer artefacts attests to the utility of these PCR products for subsequent Sanger sequencing. This validation step ensured the integrity of the amplified fragments and reduced the risk of sequencing errors caused by off-target amplification.

3.4.3 Sanger Sequencing Confirmation of Variants in HRAS, NRAS, RB1, and RET Genes

For validation and further characterization of the nucleotide variants discovered by NGS, Sanger sequencing was performed on the PCR-amplified genomic regions of the selected HRAS, NRAS, RB1, and RET genes in the DNA samples derived from papillary thyroid carcinoma (PTC) tissues. Sequencing chromatograms were analyzed by reference genome alignment (NCBI RefSeq) for visualizing every corresponding site of substitution with high resolution.

Examples of sequence alterations validated amongst six genes using the chromatograms of Figures 3.4 and 3.5 are represented. In the case of HRAS, variants found at genomic positions 6309 and 6219 are present in exon 2. In RB1, polymorphisms were detected at positions 161010, 161020, and 161076, all within exon 20. Two NRAS gene polymorphisms were also confirmed at positions 7775 and 7797, which are both located within exon 3, a mutational hotspot for activating mutations in numerous tumors.

Figure 3.4: Sanger Sequencing Chromatograms Confirming HRAS, RB1, and NRAS Gene Variants.

Figure 3.5: Sanger Sequencing Chromatograms Confirming RET Gene Variants.

In addition, Sanger sequencing also verified multiple nucleotide changes in the RET gene, which is often involved in thyroid carcinogenesis. These included variants at genomic positions 46318, 46327, 46571, 46613, and 46714 in the intronic and exonic regions that were intron 13 and exon 13, respectively. High-quality peaks covered all of those observed variants, and they were aligned to the reference sequences, leading to the successful base calling.

The high peaks, which are sharp, non-overlapping, and appear in every lane of both chromatograms, support the successful amplification and high-quality sequencing. These findings confirm the existence of somatic-specific variants in tumor tissue and suggest that the PCR-Sanger method is a reliable tool for mutation screening of PTC. The mutations, when annotated, may inform our understanding of the molecular mechanisms underlying the disease and provide insights into inter-tumor heterogeneity among patients.

3.4.4 Gene Characteristics and Genomic Context of Mutations Identified by Sanger Sequencing

Sanger sequencing of selected genomic regions of NRAS, HRAS, RB1, and the RET genes allowed the identification of a total of twelve single-nucleotide variations in the DNA of the PTC fresh tissue samples. These differentiating variants were then annotated with their corresponding genomic coordinates, position of chromosome, transcript, and exact mapping to exonic or intronic locations (Table 3.10).

Two intronic variants were found in the NRAS gene, which were discovered at chr1:114714098 (7797G>A) and chr1:114714120 (7775T>A), both of which are located in intron 2 of transcript NM_002524.5. Two exonic changes, 6309T>C and 6219C>T, in HRAS were located in exon 2 on transcript NM_005343.4. Concerning the RB1 gene, there were 3 mutations in exon 20 referred to transcript NM_000321.3, at the position of 161010G>A, 161020T>A, and 161076C>T, all on chromosome 13q14. 2.

Variants were found in RET in five SNPs. Two of them—46318G>A and 46327T>G—were inside exon 13 and 46571G>A, 46613G>A, and 46714G>A in intron 13 of the NM_020975.6 transcript. Mutations in RET were all localized in or near exon 13 on chromosome 10q11.21.

Table 3.10: Genomic Characteristics of Variants Identified by Sanger Sequencing

Gene Variant	Genomic Location	Chromosomal Location	Transcript Location	Exon/Intron number/Total number
NRAS 7797G>A	chr1:114714098	1p13.2	NM_002524.5	Intron 2/6
NRAS 7775T>A	chr1:114714120	1p13.2	NM_002524.5	Intron 2/6
HRAS 6309T>C	chr11:534242	11p15.5	NM_005343.4	Exon 2/6
HRAS 6219C>T	chr11:534332	4q12	NM_005343.4	Exon 2/6
RB1 161010G>A	chr13:48459756	13q14.2	NM_000321.3	Exon 20/27
RB1 161020T>A	chr13:48459766	13q14.2	NM_000321.3	Exon 20/27
RB1 161076C>T	chr13:48459822	13q14.2	NM_000321.3	Exon 20/27
RET 46318G>A	chr10:43118386	10q11.21	NM_020975.6	Exon 13/20
RET 46327T>G	chr10:43118395	10q11.21	NM_020975.6	Exon 13/20
RET 46571G>A	chr10:43118639	10q11.21	NM_020975.6	Intron 13/19
RET 46613G>A	chr10:43118681	10q11.21	NM_020975.6	Intron 13/19
RET 46714G>A	chr10:43118782	10q11.21	NM_020975.6	Intron 13/19

All the mutations were confirmed by aligning the sequences with their reference gene sequences. The chromosomal position and exon-intron distribution of these mutations, defined by the observed novel mutations, supply a framework for additional classification of variants and for potential genotype-phenotype correlation in the PTC cohort investigated here.

3.4.5 Mutation Characteristics and Functional Predictions of Variants Identified by Sanger Sequencing

Following re-sequencing of candidate gene regions in PTC DNA samples using Sanger technology, twelve polymorphic nucleotides were identified in NRAS, HRAS, RB1, and RET (Table 3.11). These were then evaluated for mutation type, clinical significance, and population frequency. Their predicted functional effects were assessed with in silico tools such as SIFT and PolyPhen, and compared with available data from dbSNP and COSMIC.

A graph illustrates the percentage of mutations detected in the study cohort for each variant, along with mutation frequency through Sanger sequencing shown in a bar chart (Fig. 3.6). The RET 46714G>A intronic variant was the most common, accounting for 81%. In contrast, the RET 46327T>G synonymous variant was present in 57% of cases. Mutation types are indicated by color: intronic (green), synonymous (purple), missense (blue), and 5′ UTR upstream transcript variant-new sequence (red). The prevalence of non-translated variants and the RET further highlights the regulatory or non-canonical mode of action underlying PTC onset.

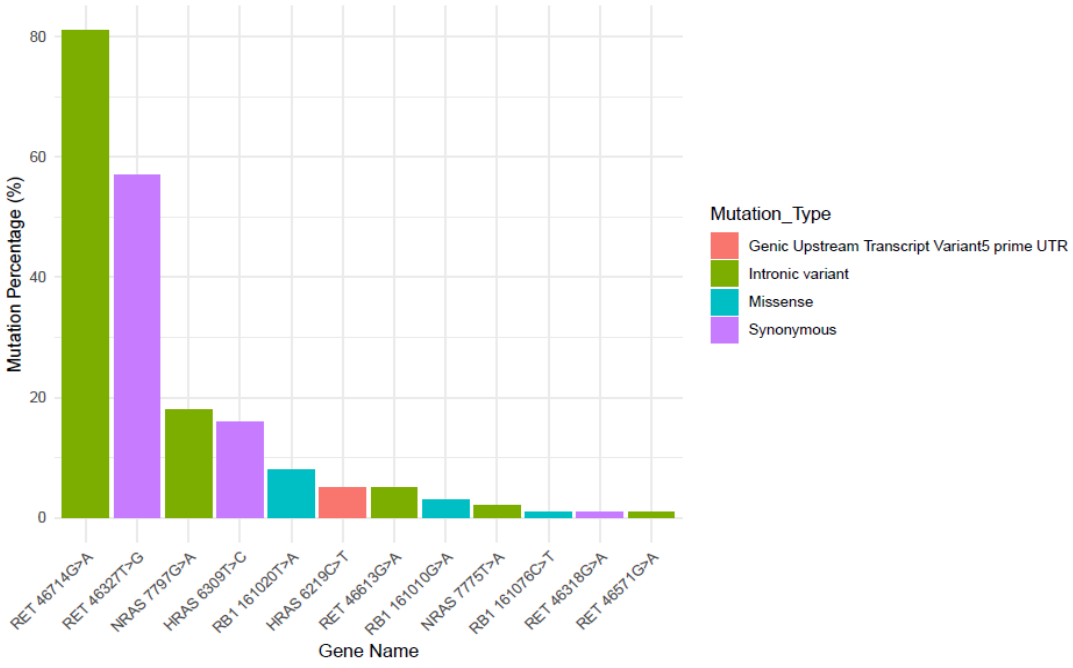


Figure 3.6: Distribution of Mutation Types and Frequencies in Genes Detected by Sanger Sequencing.

In the NRAS gene, two novel intronic variants, g.7775T>A and g.7797G>A, were detected in 2% and 18% of the cohort, respectively. Lacking previous annotation and predictive scores, these variants were categorized as "Unknown" regarding their functional impact and clinical significance.

Within the HRAS gene, two substitutions were observed. The synonymous variant c.81T>C (p.His27=)—identified as rs12628 and recorded in COSMIC—was present in 16% of patients and predicted to be benign. Another substitution, c.-10C>T, located in the 5′ untranslated region (5′ UTR) and annotated as rs41294870, was observed in 5% of patients and likewise classified as benign.

Three missense mutations were identified in the RB1 gene. The variant c.2029G>A (p.Glu677Lys), annotated as rs1060503067, appeared in 3% of cases and was predicted to be probably damaging (SIFT: 0.26; PolyPhen: 0.995). The novel variant c.2039T>A (p.Ile680Asn), present in 8% of samples, showed the highest predicted pathogenicity with SIFT: 0.00 and PolyPhen: 1.00. Similarly, c.2095C>T (p.His699Tyr), found in 1% of cases and listed as rs2138336493, was also predicted to be probably damaging with maximal pathogenicity scores from both tools.

The RET gene harbored six distinct variants. Two synonymous mutations, c.2298G>A (p.Pro766=) and c.2307T>G (p.Leu769=), were observed in 1% and 57% of the cohort, respectively. Both were annotated in dbSNP (rs140658743 and rs1800861) and COSMIC and predicted to be benign.

Among the three intronic variants in RET, c.2392+159G>A (rs3026767) and c.2392+302G>A (rs2075910) were identified in 1% and 81% of samples, respectively, with both reported as benign. The remaining intronic variant, c.2392+201G>A (rs1588876668), was found in 5% of patients and currently lacks explicit annotation; thus, it is considered functionally unknown.

In the NRAS gene, two new intronic polymorphisms, g.7775T>A and g.7797G>A, were identified and found in 2% and 18% of the patients, respectively. Since there were no prior annotations or predictive scores for these variants, they were labeled as "Unknown" in terms of functional impact and clinical significance.

Table 3.11: Mutation Characteristics and In Silico Functional Predictions of Variants Identified by Sanger Sequencing

Gene Variant	Nucleotide and amino acid change	Mutation Type	Annotation	%Mutation	SIFT Score	PolyPhen Score	Pathology Prediction
NRAS 7775T>A	g.7775T>A	Intronic variant	Novel	2%	-	-	Unknown
NRAS 7797G>A	g.7797G>A	Intronic variant	Novel	18%	-	-	Unknown
HRAS 6309T>C	c.81T>C p.His27=	Synonymous	rs12628, COSM 249860	16%	-	-	Benign
HRAS 6219C>T	c.-10C>T	Genic Upstream Transcript Variant 5 prime UTR	rs41294870, COSM 249860	5%	-	-	Benign
RB1 161010G>A	c.2029G>A p.Glu677Lys	Missense	rs1060503067	3%	0.26	0.995	Probably Damaging
RB1 161020T>A	c.2039T>A p.Ile680Asn	Missense	Novel	8%	0.00	1.00	Probably damaging
RB1 161076C>T	c.2095C>T p.His699Tyr	Missense	rs2138336493	1%	0.00	1.00	Probably damaging
RET 46318G>A	c.2298G>A p.Pro766=	Synonymous	rs140658743	1%	-	-	Benign
RET 46327T>G	c.2307T>G p.Leu769=	Synonymous	rs1800861 COSM4418405	57%	-	-	Benign
RET 46571G>A	c.2392+159G>A g.46571G>A	Intronic variant	rs3026767	1%	-	-	Benign
RET 46613G>A	c.2392+201G>A	Intronic variant	rs1588876668	5%	-	-	Unknown
RET 46714G>A	c.2392+302G>A	Intronic variant	rs2075910	81%	-	-	Benign

Two substitutions were identified within the HRAS gene. The synonymous substitution c.81T>C (p.His27=), known as rs12628 and listed in COSMIC, was found in 16% of patients and classified

as benign. Another nucleotide change, c.-10C>T, located in the 5′UTR and designated as rs41294870, was present in 5% of patients and also classified as benign.

Three missense mutations of the RB1 gene were identified. The c.2029G>A (p.Glu677Lys) variant (rs1060503067) had a minor allele frequency of 3% in cases and was predicted to be probably damaging (SIFT: 0.26, Polyphen2 HVAR: 0.995). The novel mutation c.2039T>A (p.Ile680Asn), which was the most common mutation found in 8% of cases analyzed, was considered the most deleterious by in silico prediction with SIFT 0.00 and PolyPhen 1.00. Similarly, c.2095C>T (p.His699Tyr), found in 1% of patients and described as rs2138336493, was also classified as probably damaging, with the highest pathogenicity scores by both predictors.

At the gene level, RET had a total of six variants. Two common variants, c.2298G>A (p.Pro766=) and c.2307T>G (p.Leu769=), were found in 1% and 57% of patients, respectively. Both were listed in dbSNP (rs140658743 and rs1800861) and COSMIC as benign.

Of the three intronic RET variants, c.2392+159G>A (rs3026767) and c.2392+302G > A (rs2075910) were observed in 1% and 81% of samples, respectively, both classified as benign. The other intronic variant, c.2392+201G>A (rs1588876668), is present in 5% of individuals and has no stated interpretation or annotation, so it is functionally indeterminate.

Overall, three missense mutations within RB1 exhibited high deleterious potential according to SIFT and PolyPhen predictions, while most of the RET and HRAS mutations were deemed benign or likely tolerated. Functional assays or splicing analyses are necessary to investigate the regulatory role of the novel and intronic variants of NRAS and RB1.

3.5 Evaluation of Novel NRAS and RB1 Variants in Papillary Thyroid Carcinoma Patients

3.5.1 Correlation Analysis of NRAS and RB1 Variants Using Pearson Coefficients

Figure 3.7 shows the Pearson correlation coefficients (r) and their corresponding p-values assessing the pairwise relationships between selected variants of the NRAS and RB1 genes in the

PTC study cohort. The correlation matrix on the right displays both the strength and direction of the associations, with significance levels indicated by asterisks ($p < 0.05$ as *, $p < 0.001$ as ***)

Among the tested pairs, a statistically significant moderate negative correlation was observed between NRAS 7775T>A and NRAS 7797G>A (r = –0.4330, p = 0.0271). A more notable and highly significant negative correlation was found between NRAS 7797G>A and RB1 161020T>A (r = –0.6389, p = 0.0004), indicating a possible inverse relationship in mutational occurrence within this cohort. No statistically significant correlation was detected between NRAS 7775T>A and RB1 161020T>A (r = –0.1925, p = 0.3462).

Gene variant 1	Gene variant 2	Pearson r	p-value
NRAS 7775T>A	NRAS 7797G>A	-0.4330	0.0271
NRAS 7775T>A	RB1 161020T>A	-0.1925	0.3462
NRAS 7797G>A	RB1 161020T>A	-0.6389	0.0004

Gene variant pair co-occurrence was evaluated using Pearson correlation. The results are reported as numbers. The significance level was set at $p<0.05$

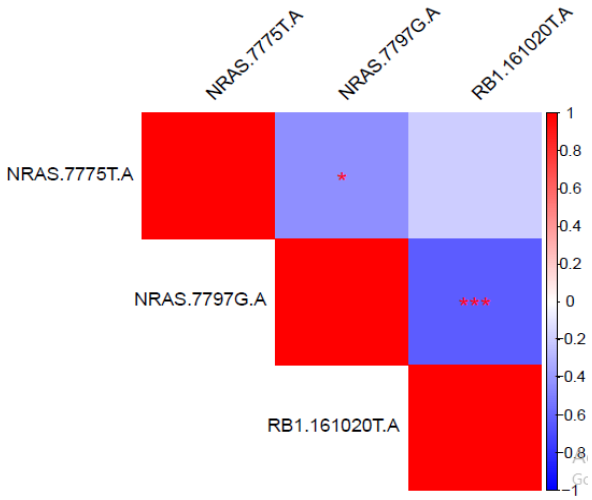


Figure 3.7: Pearson Correlation Analysis of NRAS and RB1 Gene Variants in PTC Tissue Samples.

The findings show a non-random pattern of co-occurrence, especially between NRAS and RB1 mutations. This may indicate underlying biological interactions or mutational exclusivity in specific regulatory contexts.

3.5.2 Group Stratification and Genotypic Distribution Analysis of Novel NRAS and RB1 Variants

For potential genotype–phenotype correlations, the study subjects were divided into two groups based on the presence or absence of novel NRAS or RB1 mutations detected by Sanger sequencing.

Subjects with at least one novel mutation in either gene are referred to as the “mutant group.” In contrast, participants without these mutations are classified as the “wild-type group.” This categorization allowed for comparison of genotype distributions within subgroups of key clinical and demographic variables (age, sex, smoking, family history of thyroid disorders excluding thyroid cancer, family history of thyroid cancer, and family history of other cancers).

This strategy aimed to determine whether the presence of novel variants in NRAS or RB1 genes is associated with specific clinical features or familial cancer patterns within the papillary thyroid carcinoma (PTC) cohort. Understanding these associations may provide insights into the biological significance of these mutations and their potential impact on disease risk or heterogeneity.

3.5.3 Demographic and Genetic Profiles of PTC Patients with Novel NRAS and RB1 Variants

In targeted analysis, 26 patients with at least one novel mutation in the NRAS or RB1 gene were included as the case group. The demographic distribution of this group shows females as the predominant group (n = 21, 80.8%), while male participants were few (n = 5, 19.2%). The cohort included participants aged 16 to 51 years; the group was mainly young and middle-aged (mean approximately 31 years) (Table 3.12).

The clinical stages, according to the TNM classification, were widely distributed, ranging from early to advanced stages of disease. A large portion of the patients have pT1 or pT2 tumors, meaning small or moderate-sized tumors. However, some cases (e.g., PPTC 17, PPTC 21, PPTC 106) presented pT3 stage, indicating an advanced local disease stage. N1 was present in a few of the individuals, suggesting that regional metastasis occurred in some cases of the mutant group.

From a genotypic perspective, the most common intronic variant was NRAS 7797G>A, found in 20 out of 26 patients (76.9%), either as a single mutation or in combination with other mutations. The RB1 161020T>A missense variant was identified in 9 cases (34.6%) either as a standalone mutation or coexisting with an NRAS variant. However, the latter variant was only present in 2 (7.7%) patients, with one case showing it as the only variant (PPTC 103) and another showing it alongside no other variants (PPTC 106).

Table 3.12: Demographic, Clinical, and Genotypic Profiles of the Case Group Harboring Novel NRAS and RB1 Variants

Serial No.	Sample ID	Age	Sex	Stage	NRAS 7775T>A	NRAS 7797G>A	RB1 161020T>A
1	PPTC 01	31	F	pT1NxMx	No	Yes	No
2	PPTC 02	31	M	pT1aNxMx	No	No	Yes
3	PPTC 03	39	F	pT2NxMx	No	Yes	No
4	PPTC 09	26	F	pT2NxMx	No	Yes	No
5	PPTC 10	16	F	pT2N1Mx	No	Yes	No
6	PPTC 11	23	F	pT1bNxMx	No	Yes	No
7	PPTC 16	46	F	pT2NxMx	No	Yes	No
8	PPTC 17	51	M	pT3N1Mx	No	Yes	No
9	PPTC 18	25	F	pT3N1bMx	No	Yes	No
10	PPTC 19	23	F	pT2NxMx	No	Yes	No
11	PPTC 21	39	M	pT3aNxMx	No	Yes	No
12	PPTC 27	49	F	pT1N1aMx	No	Yes	No
13	PPTC 35	45	F	pT1aN1bMx	No	No	Yes
14	PPTC 37	28	F	pT2N1Mx	No	Yes	No
15	PPTC 42	26	F	pT1N1Mx	No	No	Yes
16	PPTC 43	35	F	pT1bNxMx	No	No	Yes
17	PPTC 44	36	F	pT1NxMx	No	Yes	Yes
18	PPTC 48	26	F	pT1N0Mx	No	No	Yes
19	PPTC 57	27	F	pT2NxMx	No	Yes	No
20	PPTC 69	35	F	pT1NxMx	No	Yes	Yes
21	PPTC 83	18	F	pT2N0Mx	No	Yes	No
22	PPTC 85	26	F	pT2N1Mx	No	Yes	No
23	PPTC 88	35	F	pT3NxMx	No	No	Yes
24	PPTC 89	29	M	pT1NxMx	No	Yes	No
25	PPTC 103	39	F	pT1NxMx	Yes	No	No
26	PPTC 106	35	M	pT3aN1bMx	Yes	No	No

In summary, this cohort exhibits some genetic and clinical diversity, with specific mutations, notably NRAS 7797G > A, being prevalent. These findings support further comparison tests with wild-type cases to explore the connection between these mutations and clinicopathological features, such as tumor stage, lymph node involvement, and age at diagnosis.

3.5.4 Comparison of Age and Disease Onset between Wild-Type and Mutant Groups

To evaluate possible age-dependent differences in the clinical expression of the disease, the overall age and age at the onset of PTC were compared between the group of patients negative for mutations (wild type) and the group of patients with at least one novel NRAS or RB1 variant (mutant) (Table 3.13). Statistical comparisons were made using appropriate tests, with significance set at $p < 0.05$.

Age at the Time of Sampling

Across the entire cohort, the average age was 35.27 ± 1.167 years. When broken down by group, wild-type individuals had a slightly higher average age (36.60 ± 1.426 years) compared to the mutant group (32.27 ± 1.785 years), although this difference was not statistically significant ($p = 0.1012$). Subgroup analysis by age categories (≤25, 26–39, ≥40 years) also revealed no significant differences between the two groups ($p > 0.36$ for all).

Age at PTC Onset

When examining the onset age of PTC, a significant difference was observed. Compared to wild-type, the average age of onset in the mutant group (30.12±1.699 years) was younger than in the wild-type group (35.29±1.404 years) ($p = 0.0466$), indicating a potential earlier onset in mutation carriers. Specifically, among individuals aged 40 years and older, the mutant group had a significantly lower mean age of onset (42.75 ± 1.109) than their wild-type counterparts (50.70 ± 1.286, $p = 0.0135$). No significant differences were found in most other age categories.

These observations suggest a possible tendency for earlier disease presentation in individuals with novel NRAS or RB1 variants, potentially reflecting genetic factors that influence tumor biology, which warrants further investigation through longitudinal and mechanistic studies.

Table 3.13: Comparison of Age and Age at PTC Onset between Wild-Type and Mutant Groups

Variable	Category	Value in number (n)	Wild Type	Mutant Type	p-value
Age					
	≤25	20	21.64±0.7383	21.00±1.703	0.6900
	26-39	52	32.54±0.6154	31.94±1.205	0.6228
	≥40	28	51.25±1.521	47.75±1.377	0.3677
	Overall	35.27±1.167	36.60±1.426	32.27±1.785	0.1012
PTC Onset Age					
	≤25	22	21.55±0.6219	20.00±1.304	0.3394
	26-39	51	31.81±0.5723	31.09±0.9578	0.5253
	≥40	27	50.70±1.286	42.75±1.109	0.0135
	Overall		35.29±1.404	30.12±1.699	0.0466

The statistical significance was evaluated using a t-test. The results are reported as numbers (Mean±SEM). The significance level was set at $p<0.05$

3.5.5 Comparative Genotypic Distribution of NRAS and RB1 Variants in Wild-Type and Mutant Groups

To evaluate the significance of the observed gene variants in NRAS and RB1, a comparison of genotypic distribution was performed between the mutant group (n = 26) and the wild-type group (n = 74) (Table 3.14).

The A allele for the NRAS 7797G>A variant was only detected in mutant individuals and not in wild-type ones (n = 18). Moreover, this variant was strongly correlated with mutation status (Fisher's exact p). The A polymorphism showed a suggestive trend associated with the phenotype. Since the A allele was found only in homozygous mutant individuals (n = 2) and is absent in the wild type, the evidence was not strong enough to reach statistical significance (Fisher's p = 0.0657;

$\chi^2 = 2.547$, $p = 0.1105$). This indicates that larger sample sizes or functional validation studies are needed to determine its potential effect.

Table 3.14: Genotypic Distributions of NRAS and RB1 Variants in Wild-Type and Mutant Groups

Variant	Wild Type	Mutant Type	p-value from Fisher's exact test	χ2 (p-value)
NRAS 7797G	74	8	Ref.	Ref.
NRAS 7797A	0	18	<0.0001	57.87 (<0.0001)
NRAS 7775T	74	24	Ref.	Ref.
NRAS 7775T	0	2	0.0657	2.547 (0.1105)
RB1 161020T	74	18	Ref.	Ref.
RB1 161020A	0	8	<0.0001	20.75 (<0.0001)

The statistical significance was evaluated using Fisher's exact test and the chi-square test. The results are reported as numbers. The significance level was set at $p<0.05$.

The RB1 161020T>A variant A allele was observed only in the mutant type (n = 8) and not in the wild type, resulting in a strong and statistically significant association (Fisher's $p < 0.0001$; $\chi^2 = 20.75$, $p < 0.0001$). Both RB1 161020T>A, and the variant are considered potential candidates of biological and statistical importance related to disease status, warranting further functional and clinical analyses.

3.5.6 Gender-Based Distribution of NRAS and RB1 Gene Variants in Wild-Type and Mutant Groups

To assess whether the distribution of NRAS 7797G>A and RB1 161020T>A variants differed by sex, genotypic frequencies were compared between male and female subjects within the wild-type and mutant groups (Table 3.15).

Among male participants, the A allele of NRAS 7797G>A was found in three individuals with the mutant genotype, and it was not present in the male wild-type group. This association was statistically significant, with a p-value of 0.0038 and a chi-square value of 8.972 (p = 0.0027), indicating a strong link between the NRAS 7797A variant and mutation-positive status in males.

In contrast, among female subjects, the A allele was found in 15 mutant-type individuals, while no carriers were observed in the female wild-type group. This resulted in an even stronger statistical association ($p < 0.0001$, $\chi^2 = 27.03$). However, the odds ratio (OR = 1.189) was not statistically significant ($p > 0.9999$), mainly because there were no alternate alleles in the wild-type group, which limited the accuracy of the risk estimate.

Table 3.15: Genotypic Distribution of NRAS and RB1 Variants in Wild-Type and Mutant Groups Stratified by Gender

Variable	Gene variant	Wild Type	Mutant Type	OR (95% CI)* p-value	χ2 (p-value)
Male	NRAS 7797G	21	2	Ref.	Ref.
	NRAS 7797A	0	3	0.0038	8.972 (0.0027)
Female	NRAS 7797G	53	6	1.189 (0.2634-6.149)* >0.9999	0.045 (0.832)
	NRAS 7797A	0	15	<0.0001	27.03 (<0.0001)
Male	RB1 161020T	21	4	Ref.	Ref.
	RB1 161020A	0	1	0.1923	0.6339 (0.4259)
Female	RB1 161020T	53	14	1.387 (0.4388-4.209)* 0.7705	0.0534 (0.8172)
	RB1 161020A	0	7	<0.0001	13.58 (0.0002)
Male	Overall	21	5	Ref.	Ref.
Female	Overall	53	21	1.664 (0.5685-4.448)* 0.4425	0.4289 (0.5125)

The statistical significance was evaluated using Fisher's exact and chi-square tests. The results are reported as numbers. The significance level was set at $p<0.05$.

A similar pattern was observed for the RB1 161020A allele. Among males, only one mutant-type individual carried the variant, with none among wild types (p = 0.1923), indicating a non-

significant trend. Among females, the variant was found in seven mutant-type individuals and was absent in the wild-type group, resulting in a statistically significant finding ($p < 0.0001$ by Fisher's exact test; $\chi^2 = 13.58$, $p = 0.0002$).

When assessing the overall mutation status (i.e., presence of any NRAS or RB1 variant), 5 out of 26 males (19.2%) and 21 out of 74 females (28.4%) were classified as mutant. Although the odds ratio for females (OR = 1.664; 95% CI: 0.5685–4.448) indicated a higher mutation rate compared to males, the difference was not statistically significant ($p = 0.4425$, $\chi^2 = 0.4289$, $p = 0.5125$).

These results show that both NRAS 7797A and RB1 161020A alleles are enriched in mutation-positive individuals regardless of sex, although their occurrence seems slightly higher in females.

3.5.7 Genotypic Distribution of NRAS and RB1 Variants by Smoking Status in Wild-Type and Mutant Groups

To investigate potential associations between genetic changes and environmental exposure, especially smoking status, the distribution of NRAS 7797G>A and RB1 161020T>A variants was compared between smokers and non-smokers in both wild-type and mutant groups (Table 3.16).

Among non-smokers, the alternative A allele of NRAS 7797G>A was found in 16 individuals with the mutant type and was absent in the wild-type group. This association was highly significant ($p < 0.0001$, $\chi^2 = 46.68$), indicating a strong link between the NRAS 7797A variant and mutation-positive status among non-smokers.

Among smokers, the A allele was found in two individuals with the mutant type and was not detected in the wild-type group. Although the small sample size limited statistical power, the association remained statistically significant ($p = 0.0145$, $\chi^2 = 7.222$), indicating that the variant may also be present among smokers, though at a lower frequency.

Table 3.16: Genotypic Distribution of NRAS and RB1 Variants by Smoking Status in Wild-Type and Mutant Groups

Variable	Gene variant	Wild Type	Mutant Type	OR (95% CI)* p-value	$\chi 2$ (p-value)
Non-smoker	NRAS 7797G	62	7	Ref.	Ref.
	NRAS 7797A	0	16	<0.0001	46.68 (<0.0001)
Smoker	NRAS 7797G	12	1	0.7381 (0.6091-5.11)* >0.9999	0.05575 (0.8134)
	NRAS 7797A	0	2	0.0145	7.222 (0.0072)
Non-smoker	RB1 161020T	62	16	Ref.	Ref.
	RB1 161020A	0	7	<0.0001	16.73 (<0.0001)
Smoker	RB1 161020T	12	2	0.6458 (0.1336-2.73)* 0.7293	0.0306 (0.8611)
	RB1 161020A	0	1	0.2152	0.4865 (0.4855)
Non-smoker	Overall	62	23	Ref.	Ref.
Smoker	Overall	12	3	0.6739 (0.1898-2.532)* 0.7533	0.0652 (0.7984)

The statistical significance was evaluated using Fisher's exact and chi-square tests. The results are reported as numbers. The significance level was set at $p<0.05$

Overall, 23 of 26 mutation-positive individuals were non-smokers, with only three in the smoking group. Although the calculated odds ratio (OR = 0.6739) suggested a lower likelihood of mutation among smokers, the difference was not statistically significant ($p = 0.7533$, $\chi^2 = 0.0652$).

These findings indicate that the NRAS 7797A and RB1 161020A variants are mainly present in non-smokers, which could suggest a genetically driven pathway of tumor development independent of tobacco exposure. However, the rare cases found in smokers deserve further research with larger groups to explore potential gene-environment interactions.

3.5.8 Distribution of NRAS and RB1 Gene Variants by Family History of Non-Tumorous Thyroid Disease in Wild-Type and Mutant Groups

To assess the impact of family history on genetic mutation profiles, the distribution of NRAS 7797G>A and RB1 161020T>A variants was examined in patients with a family history of thyroid disease, excluding thyroid cancer (Table 3.17).

Among individuals without a family history of non-tumorous thyroid conditions, the A allele of NRAS 7797G>A was found in 15 mutant-type cases and was absent in wild-type individuals. This distribution showed strong statistical significance ($p < 0.0001$, $\chi^2 = 44.09$), indicating a strong association between this variant and mutation-positive status in apparently sporadic PTC cases.

In contrast, among individuals with a positive family history of thyroid disease, the A allele was found in three mutant-type cases, again not present in wild types. This also had statistical significance ($p = 0.0028$, $\chi^2 = 11.2$); however, interpretation is limited by the relatively small sample size in this subgroup.

A similar trend was observed for the RB1 161020A variant. In the family history-negative group, the allele was found in seven mutant-type individuals, with none among wild types, demonstrating strong significance ($p < 0.0001$, $\chi^2 = 16.46$). Within the family history-positive group, only one mutant-type case carried the A allele, and none were observed in the wild-type group. Although the direction of effect was consistent, this difference did not reach statistical significance ($p = 0.2179$, $\chi^2 = 0.4728$), likely due to limited sample size.

When examining overall mutation status (i.e., presence of either the NRAS or RB1 variant), 22 mutant-type individuals were identified among those without a family history, compared to 4 in the family history-positive group. This distribution showed no statistically significant difference ($p > 0.9999$, $\chi^2 = 0.0024$), and the odds ratio (OR = 0.8531; 95% CI: 0.2803–2.717) did not indicate a strong association.

Table 3.17: Genotypic Distribution of NRAS and RB1 Variants by Family History of Non-Tumorous Thyroid Disease in Wild-Type and Mutant Groups

Variable	Gene variant	Wild Type	Mutant Type	OR (95% CI)* p-value	$\chi 2$ (p-value)
No	NRAS 7797G	61	8	Ref.	Ref.
	NRAS 7797A	0	15	<0.0001	44.09 (<0.0001)
Yes	NRAS 7797G	13	0	0.3441	0.6129 (0.4337)
	NRAS 7797A	0	3	0.0028	11.2 (0.0008)
No	RB1 161020T	61	16	Ref.	Ref.
	RB1 161020A	0	7	<0.0001	16.46 (<0.0001)
Yes	RB1 161020T	13	2	0.5865 (0.1221-2.425)* 0.7267	0.0957 (0.7571)
	RB1 161020A	0	1	0.2179	0.4728 (0.4917)
No	Overall	61	22	Ref.	Ref.
Yes	Overall	13	4	0.8531 (0.2803-2.717)* >0.9999	0.0024 (0.9613)

The statistical significance was evaluated using Fisher's exact test and the chi-square test. The results are reported as numbers. The significance level was set at $p<0.05$

Taken together, these results suggest that germline NRAS A7797 and RB1 A161020 variants are more prevalent in sporadic PTC, given the absence of a family history of thyroid conditions, indicating a largely non-inherited mutational landscape of PTC in this cohort.

3.5.9 Distribution of NRAS and RB1 Gene Variants by Family History of Thyroid Tumor in Wild-Type and Mutant Groups

To explore the potential genetic predisposition associated with familial thyroid tumors, the distribution of NRAS 7797G>A and RB1 161020T>A variants was assessed in individuals with

and without a family history of thyroid tumors, categorized into wild-type and mutant-type groups based on the presence or absence of mutations (Table 3.18).

To investigate the genetic predisposition for familial thyroid tumors, the distribution of the NRAS 7797G>A and RB1 161020T>A variants was assessed among the wild-type and mutant-type groups based on the presence or absence of the mutation (Table 3.18).

Among participants with no family history of thyroid tumor, the NRAS 7797A allele was present in 11 individuals with a mutant-type genotype. It was completely absent in the wild-type group, indicating a strong association with mutation status ($p < 0.0001$, $\chi^2 = 46.02$).

In individuals with a family history, the A allele was found in 7 mutant-type individuals and was absent in the wild-type group. Although the odds ratio was elevated (OR = 4.333; 95% CI: 0.9874–22.19), statistical significance was not reached with Fisher's exact test ($p = 0.0887$), likely due to the limited sample size. However, the chi-square test remained highly significant ($\chi^2 = 34.95$, $p < 0.0001$), indicating a strong association between the A allele and a familial history of thyroid tumors.

For the RB1 161020A allele, among individuals without a family history, four mutant-type individuals carried the variant, while none were found in the wild-type group, resulting in significant findings ($p = 0.0011$, $\chi^2 = 12.18$). Similarly, in the group with a family history, the A allele was present in 4 mutant-type individuals and absent in wild-type individuals ($p = 0.0011$, $\chi^2 = 12.18$). The corresponding OR (3.611; 95% CI: 0.9806–10.93) was close to significance ($p = 0.0678$), suggesting a potential trend toward familial enrichment.

When assessing overall mutation status, 10 out of 19 individuals with a family history of thyroid tumors were in the mutant-type group, compared to 16 out of 81 without a family history. This difference was statistically significant ($p = 0.0071$, $\chi^2 = 7.022$), and the odds of being mutation-positive were more than four times higher among those with a familial thyroid tumor background (OR = 4.514; 95% CI: 1.624–13.19).

Table 3.18: Genotypic Distribution of NRAS and RB1 Variants by Family History of Thyroid Tumor in Wild-Type and Mutant Groups

Variable	Gene variant	Wild Type	Mutant Type	p-value from Fisher's exact test	$\chi 2$ (p-value)
No	NRAS 7797G	65	5	Ref.	Ref.
	NRAS 7797A	0	11	<0.0001	46.02 (<0.0001)
Yes	NRAS 7797G	9	3	4.333 (0.9874-22.19)* 0.0887	1.959 (0.1616)
	NRAS 7797A	0	7	<0.0001	34.95 (<0.0001)
No	RB1 161020T	65	12	Ref.	Ref.
	RB1 161020A	0	4	0.0011	12.18 (0.0005)
Yes	RB1 161020T	9	6	3.611 (0.9806-10.93)* 0.0678	3.331 (0.068)
	RB1 161020A	0	4	0.0011	12.18 (0.0005)
No	Overall	65	16	Ref.	Ref.
Yes	Overall	9	10	4.514 (1.624-13.19)* 0.0071	7.022 (0.008)

The statistical significance was evaluated using Fisher's exact test and the chi-square test. The results are reported as numbers. The significance level was set at $p<0.05$

Although NRAS and RB1 variant frequencies were observed across both family-history positive and negative cohorts, the most pronounced statistical associations—reflected by the highest chi-square values—were found in individuals without a familial predisposition. This indicates that these mutations may define a sporadic molecular subtype of papillary thyroid carcinoma (PTC) with region-specific significance.

3.5.10 Distribution of NRAS and RB1 Gene Variants by Family History of Non-Thyroid Cancers in Wild-Type and Mutant Groups

To evaluate whether a broader family history of cancer, beyond thyroid malignancies, influences the prevalence of gene mutations, the distribution of NRAS 7797G>A and RB1 161020T>A

variants was analyzed among wild-type and mutant groups, stratified by the presence or absence of a family history of non-thyroid cancer (Table 3.19).

Among individuals without a family history of non-thyroid cancer, 11 mutant-type individuals carried the NRAS 7797A allele. In contrast, none of the wild-type participants did, resulting in highly significant results ($p < 0.0001$, $\chi^2 = 47.17$).

Table 3.19: Genotypic Distribution of NRAS and RB1 Variants by Family History of Non-Thyroid Cancers in Wild-Type and Mutant Groups

Variable	Gene variant	Wild Type	Mutant Type	OR (95% CI)* p-value	χ2 (p-value)
No	NRAS 7797G	57	3	Ref.	Ref.
	NRAS 7797A	0	11	<0.0001	47.17 (<0.0001)
Yes	NRAS 7797G	17	5	5.588 (1.232-22.23)* 0.0293	3.909 (0.048)
	NRAS 7797A	0	7	<0.0001	37.39 (<0.0001)
No	RB1 161020T	57	12	Ref.	Ref.
	RB1 161020A	0	2	0.0366	3.973 (0.0462)
Yes	RB1 161020T	17	6	1.676 (0.5182-4.914)* 0.3745	0.3684 (0.5439)
	RB1 161020A	0	6	<0.0001	16.37 (<0.0001)
No	Overall	57	14	Ref.	Ref.
Yes	Overall	17	12	2.874 (1.114-7.371)* 0.0426	3.958 (0.0466)

The statistical significance was evaluated using Fisher's exact test and the chi-square test. The results are reported as numbers. The significance level was set at $p<0.05$

For individuals with a positive family history, the A allele was also more common in 7 mutant-type cases, with none found in the wild-type group. This correlation remained statistically significant ($p < 0.0001$, $\chi^2 = 37.39$). Furthermore, the odds of carrying the A allele were more than five times higher in this group (OR = 5.588; 95% CI: 1.232–22.23; $p = 0.0293$), suggesting a strong link between family cancer history and the presence of NRAS variants.

Regarding the RB1 161020A allele, among participants without a family history, it was found in 2 mutant-type individuals and was absent from the wild-type group ($p = 0.0366$, $\chi^2 = 3.973$). In the family-history-positive group, the A allele appeared in 6 mutant-type individuals and was absent in the wild-type individuals, resulting in a highly significant result ($p < 0.0001$, $\chi^2 = 16.37$). However, the OR (1.676; 95% CI: 0.5182–4.914) was not statistically significant ($p = 0.3745$), likely due to sample size limitations.

When comparing overall mutation status, 12 of 29 individuals with a family history of non-thyroid cancer were in the mutant-type group, compared to 14 of 71 without such a history. This resulted in a statistically significant difference ($p = 0.0426$, $\chi^2 = 3.958$), with an OR of 2.874 (95% CI: 1.114–7.371), indicating that patients with a family history of non-thyroid cancer are nearly three times more likely to carry germline variants in NRAS or RB1.

These findings emphasize a possible familial predisposition beyond thyroid-specific cancers, indicating that NRAS and RB1 germline variants may be associated with a wider oncogenic susceptibility within affected families.

3.5.11 Distribution of NRAS and RB1 Variants by Tumor Size and Metastatic Classification in Wild-Type and Mutant Groups

To explore the relationships between tumor burden and mutation status, the genotypic distributions of NRAS 7797G>A and RB1 161020T>A variants were analyzed across four clinically relevant subgroups of papillary thyroid carcinoma (PTC): small tumors (T1), moderate tumors without nodal involvement (T2, N0), advanced tumors (T3), and PTC with lymph node (LN) metastasis (Table 3.20).

NRAS 7797G>A Variant Distribution

Small PTC (T1): The NRAS 7797A allele was present in 6 mutant-type individuals and absent in all wild-type individuals, leading to a statistically significant enrichment ($p = 0.0002$, $\chi^2 = 13.65$).

Moderate PTC (T2, N0): The A allele was also found in six mutant-type cases and was not present among wild-types ($p = 0.0002$, $\chi^2 = 13.65$), although the wild-type sample size was smaller, which resulted in a non-significant OR for the G allele ($p = 0.1601$).

Advanced PTC (T3): The A allele was present in 3 mutant-type individuals and absent in wild-types ($p = 0.0092$, $\chi^2 = 6.647$). However, the overall difference in G carriers between wild-type and mutant-type groups was not statistically significant (OR = 0.5882; $p = 0.7$), indicating potential heterogeneity.

PTC with LN Metastasis: A significant enrichment of the A allele was observed in mutant-type individuals ($p < 0.0001$, $\chi^2 = 16.37$), with none of the wild-type individuals carrying the variant.

RB1 161020T>A Variant Distribution

Small PTC (T1): The A allele was found in 7 mutant-type patients and in none of the wild-type, showing a significant enrichment ($p < 0.0001$, $\chi^2 = 17.01$).

Moderate PTC (T2, N0): The wild-type group included 6 individuals with the mutant T allele, and no A allele was detected. Statistical analysis showed no significant association (OR = 2.25; $p = 0.3053$).

Advanced PTC (T3): A single A allele was found in mutant-type cases, with none in wild-type. This difference was not statistically significant ($p = 0.1667$, $\chi^2 = 0.8229$).

PTC with LN metastasis: The T allele was present in both groups, with no significant difference (OR = 1.636; $p = 0.6725$), and no individuals carried the A allele in this subgroup.

Compared to the T1 baseline group, the distribution of overall mutation status (NRAS and RB1 combined) did not significantly differ in moderate PTC (OR = 0.9375), advanced PTC (OR = 0.7353), or PTC with LN metastasis (OR = 0.6818), all with $p > 0.7$.

Table 3.20: Genotypic Distribution of NRAS and RB1 Variants by Papillary Thyroid Carcinoma (PTC) Classification by Tumor Size and Metastatic State in Wild-Type and Mutant Groups

Variable	Gene variant	Wild Type	Mutant Type	p-value from Fisher's exact test	$\chi 2$ (p-value)
Small PTC (T1)	NRAS 7797G	30	6	Ref.	Ref.
	NRAS 7797A	0	6	0.0002	13.65 (0.0002)
Moderate PTC (T2, N0)	NRAS 7797G	16	0	0.1601	1.603 (0.2055)
	NRAS 7797A	0	6	0.0002	13.65 (0.0002)
Advanced PTC (T3)	NRAS 7797G	17	2	0.5882 (0.1119-2.836)* 0.7	0.0449 (0.8321)
	NRAS 7797A	0	3	0.0092	6.647 (0.0099)
PTC + LN Metastasis	NRAS 7797G	11	0	0.3745	0.3684 (0.5439)
	NRAS 7797A	0	3	<0.0001	16.37 (<0.0001)
Small PTC (T1)	RB1 161020T	30	5	Ref.	Ref.
	RB1 161020A	0	7	<0.0001	17.01 (<0.0001)
Moderate PTC (T2, N0)	RB1 161020T	16	6	2.25 (0.6402-8.075)* 0.3053	0.7479 (0.3871)
	RB1 161020A	0	0	>0.9999	-
Advanced PTC (T3)	RB1 161020T	17	4	1.412 (0.3885-5.808)* 0.715	0.0088 (0.9252)
	RB1 161020A	0	1	0.1667	0.8229 (0.3643)
PTC + LN Metastasis	RB1 161020T	11	3	1.636 (0.3789-8.298)* 0.6725	0.0336 (0.8545)
	RB1 161020A	0	0	>0.9999	
Small PTC (T1)	Overall	30	12	Ref.	Ref.
Moderate PTC (T2, N0)	Overall	16	6	0.9375 (0.2746-2.951)* >0.9999	0.0335 (0.8549)
Advanced PTC (T3)	Overall	17	5	0.7353 (0.2445-2.45)* 0.7685	0.0419 (0.8377)
PTC + LN Metastasis	Overall	11	3	0.6818 (0.1797-2.695)* 0.7364	0.0304 (0.8617)

The statistical significance was evaluated using Fisher's exact test and the chi-square test. The results are reported as numbers. The significance level was set at $p<0.05$

Although statistically significant associations were observed between NRAS and RB1 variants and smaller tumor classifications, no clear trend was established across the increasing tumor burden categories. These findings suggest that while early-stage tumors may harbor detectable germline variants, mutation status does not necessarily increase with tumor aggressiveness in this cohort.

3.5.12 Distribution of NRAS and RB1 Variants by Risk Group Classification in Wild-Type and Mutant Groups

To assess potential links between mutation status and recurrence risk, the genotypic distributions of NRAS 7797G>A and RB1 161020T>A were examined across patient groups classified as low, intermediate, and high risk based on the American Thyroid Association (ATA) risk stratification guidelines (Table 3.21).

NRAS 7797G>A Variant Distribution

Among intermediate-risk individuals (n = 91), 17 mutant-type subjects carried the NRAS 7797A allele, while no such variant was found in the wild-type group. This resulted in a highly significant association ($p < 0.0001$, $\chi^2 = 50.8$), showing strong enrichment of the A allele in the mutant group.

In the high-risk group (n = 7), only one individual with the mutant type carried the A allele, while all wild-type participants tested negative for this variant. Although not statistically significant ($p = 0.12$), the OR was higher (1.386; 95% CI: not available), indicating a potentially essential but underpowered association.

There were no statistically significant differences observed across risk groups for the NRAS 7797G allele.

RB1 161020T>A Variant Distribution

In the intermediate-risk group, 8 individuals with the mutant type carried the RB1 161020A allele, while none of the wild-type counterparts had this variant. This distribution was statistically

significant ($p < 0.0001$, $\chi^2 = 19.34$), indicating a strong link between RB1 mutation and the intermediate-risk classification.

In contrast, no A alleles were found in either wild-type or mutant-type individuals in the high-risk group, suggesting this variant does not contribute to that category ($p > 0.9999$).

Table 3.21: Genotypic Distribution of NRAS and RB1 Variants by Risk Group Classification in Wild-Type and Mutant Groups

Risk Group	Gene variant	Wild Type	Mutant Type	OR (95% CI)* p-value	χ2 (p-value)
Intermediate	NRAS 7797G	66	8	Ref.	Ref.
	NRAS 7797A	0	17	<0.0001	50.8 (<0.0001)
High	NRAS 7797G	6	0	>0.9999	0.02 (0.8875)
	NRAS 7797A	0	1	0.12	1.386 (0.2391)
Intermediate	RB1 161020T	66	17	Ref.	Ref.
	RB1 161020A	0	8	<0.0001	19.34 (<0.0001)
High	RB1 161020T	6	0	0.5907	0.4827 (0.4872)
	RB1 161020A	0	0	>0.9999	-
Intermediate	Overall	66	25	Ref.	Ref.
High	Overall	6	1	0.44 (0.0369-3.005)* 0.6713	0.1007 (0.751)
Low	Overall	2	0	>0.9999	0.0037 (0.9516)

The statistical significance was evaluated using Fisher's exact test and the chi-square test. The results are reported as numbers. The significance level was set at $p<0.05$

Overall Mutation Status across Risk Groups

Out of the 25 mutant-type individuals in the intermediate-risk group, the overall frequency was significantly higher compared to the high-risk group, which had only one mutant-type case

detected (OR = 0.44; 95% CI: 0.0369–3.005; p = 0.6713). However, this result was not statistically significant due to the small size of the high-risk subgroup.

The low-risk group (n = 2) included only wild-type individuals, with no NRAS or RB1 variants detected; therefore, no statistical significance could be compared.

These findings reinforce that both NRAS 7797A and RB1 161020A variants are significantly enriched in intermediate-risk PTC patients, indicating that these variants may be more strongly linked to moderate recurrence potential rather than aggressive or indolent disease behavior. Although the high- and low-risk groups had smaller sample sizes, the findings offer valuable preliminary insights that merit further investigation with larger cohorts.

3.6 Serum Biochemical Profiling of Study Participants

3.6.1 Comparative Analysis of Biochemical Parameters among Study Groups

To examine the biochemical profile differences across various thyroid-related health states, we compared serum levels of several essential biomarkers—TSH, calcium, parathyroid hormone, thyroglobulin (Tg), and vitamin D—among three study groups: healthy individuals (Group A, n = 100), patients with non-tumorous thyroid disease (Group B, n = 100), and thyroid cancer patients (Group C, n = 100) (Table 3.22). The results are shown as mean ± SEM, and statistical significance was assessed using ANOVA followed by pairwise adjusted p-values for multiple comparisons (Figure 3.8).

Serum TSH levels showed a significant and dramatic increase across the groups ($p < 0.001$). Healthy individuals had a baseline mean of 2.167±0.107 ng/mL, while patients with non-tumorous thyroid disease and thyroid cancer had substantially higher levels of 39.33±2.492 ng/mL and 86.81±4.084 ng/mL, respectively. All pairwise comparisons (healthy vs. non-tumorous thyroid disease, healthy vs. thyroid cancer, non-tumorous thyroid disease vs. thyroid cancer) were highly significant (adjusted $p < 0.001$), confirming that TSH levels increase progressively with disease severity.

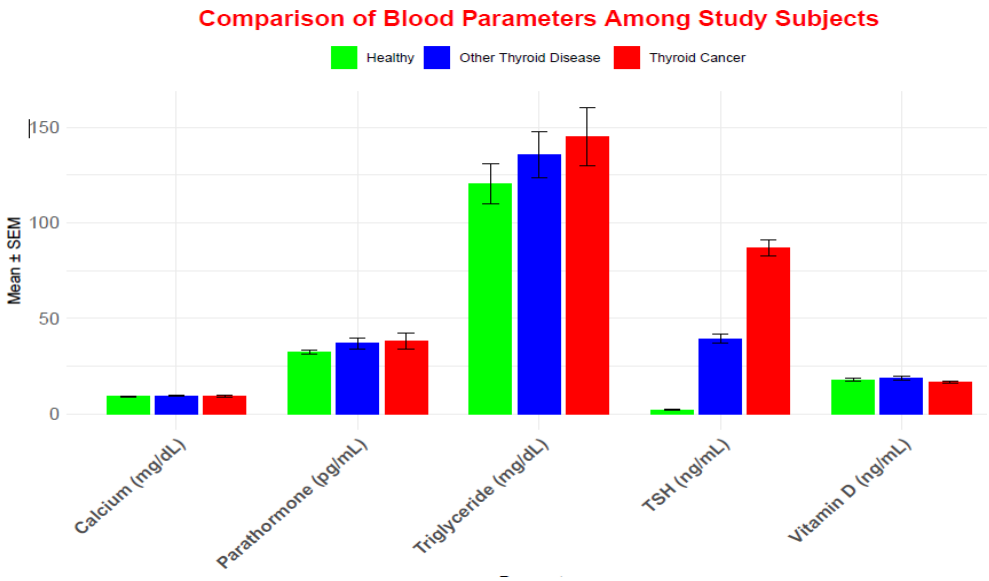


Figure 3.8: Comparison of Serum Biochemical Parameters among Study Groups.

Serum calcium levels also exhibited a significant difference among groups (p = 0.0032), with a slight decrease in thyroid cancer patients (8.387 ± 0.077 mg/dL) compared to healthy individuals (8.663 ± 0.062 mg/dL) and those with non-tumorous thyroid conditions (8.777 ± 0.104 mg/dL). The comparison between healthy and thyroid cancer groups was close to significance (adjusted p = 0.0509), and a statistically significant difference was found between the non-tumorous thyroid disease and thyroid cancer groups (adjusted p = 0.0029).

Parathyroid hormone (PTH) levels, although slightly elevated in disease groups (36.88±2.809 pg/mL in non-tumorous thyroid disease and 38.20±4.327 pg/mL in thyroid cancer), did not show statistically significant differences among the groups (p = 0.3589), indicating limited discriminatory value for this marker in this cohort.

Thyroglobulin (Tg) levels were higher in patients with thyroid-related issues, with non-tumorous thyroid disease and thyroid cancer cases showing elevated mean values (120.6±58.26 ng/mL and 93.92±44.05 ng/mL, respectively) compared to healthy individuals (4.475±0.657 ng/mL). However, because of high variability among individuals, these differences did not reach statistical significance (p = 0.114).

Table 3.22: Comparative Analysis of Serum Biochemical Parameters among Study Groups

Variable	Group A	Group B	Group C	p-value	Adjusted p-value	Adjusted p-value	Adjusted p-value
	Healthy (n=100) (Mean±SEM)	Non-Tumorous Thyroid Disease (n=100) (Mean±SEM)	Thyroid Cancer (n=100) (Mean±SEM)	(A-C)	(A vs B)	(A vs C)	(B vs C)
TSH (ng/mL)	2.167±0.107	39.33±2.492	86.81±4.084	<0.001	<0.001	<0.001	<0.001
Calcium (mg/dL)	8.663±0.062	8.777±0.104	8.387±0.077	0.0032	0.5923	0.0509	0.0029
Parathor-mone (pg/mL)	32.32±1.088	36.88±2.809	38.20±4.327	0.3589	0.5396	0.3599	0.9496
Thyroglo-bulin (Tg) (ng/mL)	4.475±0.657	120.6±58.26	93.92±44.05	0.114	0.1122	0.3054	0.8992
Vitamin D (ng/mL)	17.95±0.768	18.82±0.913	16.56±0.621	0.1192	0.7012	0.4127	0.1020

The statistical significance was evaluated using a one-way ANOVA test. The results are reported as numbers (Mean±SEM). The significance level was set at $p<0.05$

Vitamin D levels were generally similar across the groups, with a slight trend toward lower levels in patients with thyroid cancer (16.56 ± 0.621 ng/mL) compared to healthy controls (17.95 ± 0.768 ng/mL) and individuals with non-tumorous thyroid disease (18.82 ± 0.913 ng/mL). These differences were not statistically significant (p = 0.1192), although the trend might merit further investigation in a larger group.

Overall, TSH proved to be the most reliable biomarker for distinguishing between the groups, with modest yet noteworthy trends in calcium and Tg levels, indicating their potential usefulness in biochemical profiling of thyroid disorders and cancers.

3.6.2 Comparative Analysis of Biochemical Parameters between Wild-Type and Mutant Groups

This analysis compares key serum biochemical parameters between patients with mutant-type and wild-type thyroid cancer (Table 3.23). Although mean serum TSH levels were slightly higher in the wild-type group (87.94 ± 5.02 ng/mL) compared to the mutant type group (79.83 ± 4.47 ng/mL), this difference was not statistically significant (p = 0.2538). In contrast, a significant difference was observed in serum calcium levels, where the wild-type group exhibited a higher mean value (9.04 ± 0.13 mg/dL) compared to the mutant type group (8.51 ± 0.16 mg/dL), with p = 0.0305, suggesting a potential alteration in calcium metabolism associated with mutation status.

Other parameters, including parathyroid hormone, thyroglobulin (Tg), and vitamin D levels, showed no statistically significant differences between the groups. Although the wild-type group had higher average values for both parathyroid hormone (40.52 ± 5.66 pg/mL) and Tg (117.1 ± 60.70 ng/mL) compared to the mutant group (31.59 ± 4.11 pg/mL and 33.46 ± 9.32 ng/mL, respectively), the variability and broad confidence intervals likely contributed to the lack of statistical significance (p = 0.3679 for parathyroid hormone and p = 0.3988 for Tg). Vitamin D levels were almost the same between the two groups (p = 0.9756), indicating no mutation-related trend.

Table 3.23: Comparative Analysis of Serum Biochemical Parameters among Wild-Type and Mutant Groups

Variable	Wild Type (Mean±SEM)	Mutant Type (Mean±SEM)	p-value
TSH (ng/mL)	87.94± 5.018	79.83± 4.466	0.2538
Calcium (mg/dL)	9.042±0.1304	8.514±0.1621	0.0305
Parathyroid hormone (pg/mL)	40.52±5.656	31.59±4.111	0.3679
Thyroglobulin (Tg) (ng/mL)	117.1±60.70	33.46±9.319	0.3988
Vitamin D (ng/mL)	16.67±0.7889	16.63±0.9820	0.9756

The statistical significance was evaluated using a t-test. The results are reported as numbers (Mean±SEM). The significance level was set at $p<0.05$.

These findings indicate that, among thyroid cancer patients, only serum calcium levels significantly differed between those with and without NRAS/RB1 variants, potentially reflecting underlying molecular influences on calcium regulation.

3.6.3 Differences in Biochemical Profiles between Wild-Type and Mutant-Type PTC Patients Stratified by Tumor Size and Metastatic Status

To further investigate the biochemical implications of NRAS and RB1 mutational status in papillary thyroid carcinoma (PTC) patients, serum parameters were compared between wild-type and mutant-type individuals, stratified according to tumor size and lymph node involvement (Table 3.24).

Across all tumor categories—small (T1), moderate (T2 without lymph node metastasis), advanced (T3), and PTC with lymph node (LN) metastasis—there were no statistically significant differences in TSH levels between wild-type and mutant-type groups. Although wild-type individuals consistently showed slightly higher mean TSH levels, the differences did not reach significance (p-values ranging from 0.3613 to 0.9533). The overall comparison similarly revealed no significant variation ($p = 0.2538$).

Serum calcium levels exhibited greater variation. In advanced PTC patients (T3), calcium levels were significantly lower in the mutant-type group (8.03 ± 0.53 mg/dL) compared to the wild types (8.97 ± 0.19 mg/dL; $p = 0.0440$), indicating a possible disruption of calcium metabolism in mutation carriers with more aggressive disease. Although other subgroups showed similar patterns, these differences were not statistically significant. The overall comparison between wild-type and mutant-type individuals also revealed a significant decrease in calcium levels among mutant types ($p = 0.0305$).

PTH levels did not differ significantly across tumor subtypes or between mutation groups. Although elevated mean PTH was observed in wild-type individuals with LN metastasis (64.44 ± 18.91 pg/mL), variability was high, and no group reached statistical significance (all $p > 0.3$).

Table 3.24: Comparative Analysis of Serum Biochemical Parameters between Wild-Type and Mutant PTC Patients Stratified by Tumor Size and Lymph Node Metastasis

Variable	Group	Wild Type (Mean±SEM)	Mutant Type (Mean±SEM)	p-value
TSH (ng/mL)	Small PTC (T1)	87.65±7.850	81.40±9.478	0.6535
	Moderate PTC (T2, N0)	98.55±11.13	78.88±17.31	0.3613
	Advanced PTC (T3)	85.53±11.02	84.26±16.07	0.9533
	PTC + LN Metastasis	70.97±11.51	79.82±17.06	0.7191
	Overall	87.94± 5.018	79.83± 4.466	0.2538
Calcium (mg/dL)	Small PTC (T1)	9.227±0.1658	8.758±0.1855	0.1113
	Moderate PTC (T2, N0)	8.726±0.4470	8.803±0.2267	0.9195
	Advanced PTC (T3)	8.971±0.1852	8.028±0.5266	0.0440
	PTC + LN Metastasis	9.106±0.2692	8.437±0.4845	0.2675
	Overall	9.042±0.1304	8.514±0.1621	0.0305
Parathyroid hormone (pg/mL)	Small PTC (T1)	37.54±10.90	33.40±4.200	0.8145
	Moderate PTC (T2, N0)	38.35±5.878	28.71±11.80	0.4292
	Advanced PTC (T3)	32.36±7.203	33.55±14.23	0.9391
	PTC + LN Metastasis	64.44±18.91	26.89±6.550	0.3354
	Overall	40.52±5.656	31.59±4.111	0.3679
Thyroglobulin (Tg) (ng/mL)	Small PTC (T1)	47.50±28.06	40.15±15.41	0.8670
	Moderate PTC (T2, N0)	57.39±22.52	17.90±9.239	0.3053
	Advanced PTC (T3)	293.0±233.0	27.06±19.50	0.5716
	PTC + LN Metastasis	96.77±85.49	36.02±29.74	0.6857
	Overall	117.1±60.70	33.46±9.319	0.3988
Vitamin D (ng/mL)	Small PTC (T1)	18.69±1.251	16.78±1.586	0.3958
	Moderate PTC (T2, N0)	13.93±0.9407	17.46±1.133	0.0507
	Advanced PTC (T3)	16.18±1.163	14.61±1.383	0.5019
	PTC + LN Metastasis	15.90±3.256	14.36±0.554	0.8145
	Overall	16.67±0.7889	16.63±0.9820	0.9756

The statistical significance was evaluated using a t-test. The results are reported as numbers (Mean±SEM). The significance level was set at $p<0.05$.

Across all tumor categories, serum Tg levels were generally higher in the wild-type group compared to the mutant type. For example, patients with advanced PTC exhibited a notable Tg

difference (293.0 ± 233.0 ng/mL in wild type vs. 27.06 ± 19.50 ng/mL in mutant). However, the difference was not statistically significant due to variability ($p = 0.5716$). The overall comparison also was not significant ($p = 0.3988$).

Vitamin D levels were similar between wild-type and mutant-type individuals across tumor categories. A near-significant trend was observed in moderate PTC (T2, N0), where mutant-type patients had higher vitamin D levels than wild-type patients ($p = 0.0507$). However, no other group showed notable differences, and the overall comparison remained nonsignificant ($p = 0.9756$).

Although no statistically significant differences were observed in TSH, PTH, thyroglobulin, or vitamin D levels across most subgroups, trends indicated that biochemical changes might be more evident in certain tumor stages and mutation backgrounds. These findings highlight the possible connection between calcium imbalance and mutational burden in aggressive PTC phenotypes.

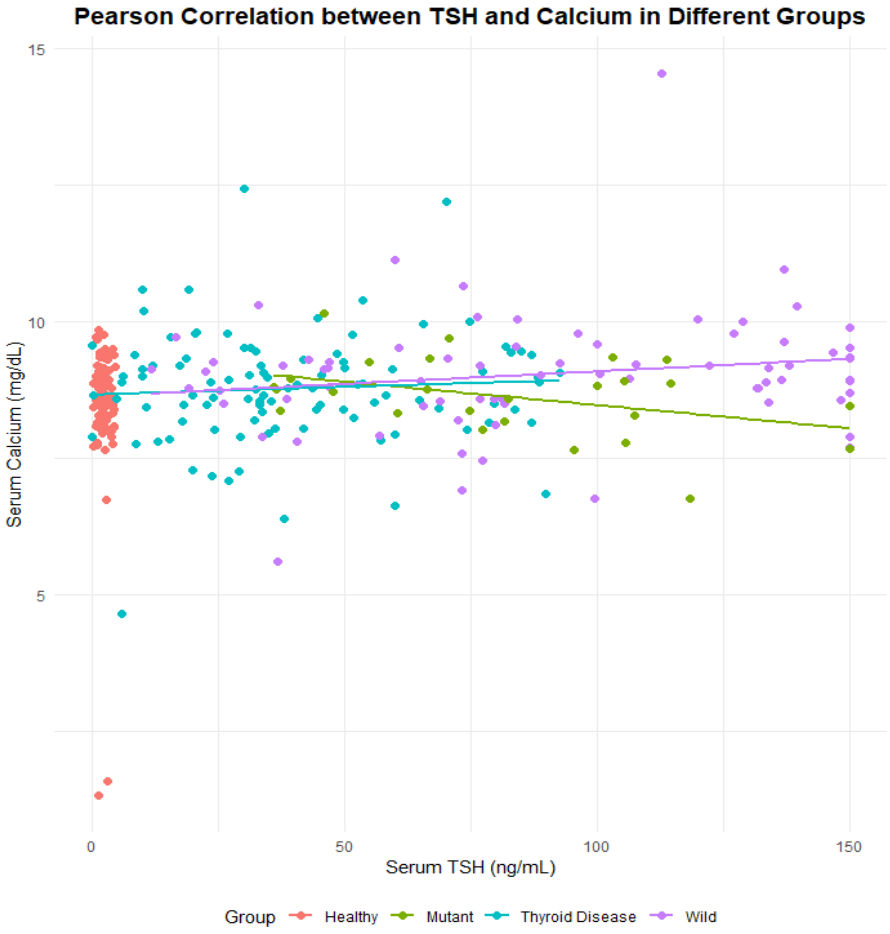
3.6.4 Correlation Analysis of Biochemical Parameters among Study Groups

3.6.4.1 Correlation Analysis between Serum TSH and Calcium Levels across Study Groups

To examine the relationship between thyroid function and calcium homeostasis, Pearson correlation analysis was performed between serum thyroid-stimulating hormone (TSH) and calcium levels across four distinct subject groups: healthy controls, individuals with non-tumorous thyroid disease, wild-type thyroid cancer patients, and mutant-type thyroid cancer patients. The scatter plot illustrates this relationship with regression trendlines for each group (Figure 3.9).

In the mutant group, a statistically significant negative correlation was observed (Pearson $r = -0.3989$, $p = 0.0435$), indicating that higher TSH levels were linked to lower calcium concentrations in patients carrying novel mutations (NRAS 7775T>A, NRAS 7797G>A, or RB1 161020T>A). This suggests a possible dysregulation of calcium metabolism in mutation-positive PTC patients.

Conversely, no statistically significant correlations between serum TSH and calcium levels were observed in the other groups. Among healthy individuals, the correlation was negligible and negative (r = –0.01908, p = 0.8506), indicating no meaningful relationship. Patients with non-tumorous thyroid disease showed a weak positive correlation (r = 0.06698, p = 0.5079), while wild-type PTC patients exhibited a slightly stronger positive trend (r = 0.1764); however, this also did not reach statistical significance (p = 0.1327).



Group	Pearson r	p-value
Healthy	-0.01908	0.8506
Thyroid Disease	0.06698	0.5079
Wild Type	0.1764	0.1327
Mutant Type	-0.3989	0.0435

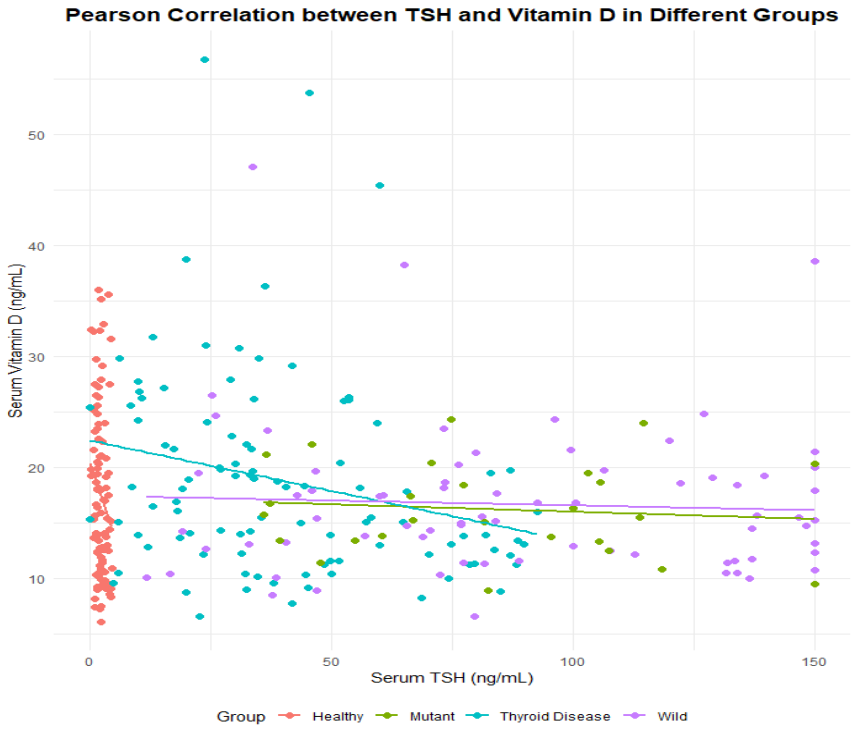
Figure 3.9: Correlation Analysis of Serum TSH and Calcium Levels among Study Groups.

Together, these findings suggest a mutation-specific disruption in calcium regulation linked to elevated TSH in the mutant group.

3.6.4.2 Correlation Analysis between Serum TSH and Vitamin D Levels across Study Groups

Among patients with non-tumorous thyroid disease, a statistically significant negative correlation was observed (r = –0.2472, p = 0.0136), indicating an inverse relationship between elevated TSH and lower vitamin D levels in this group (Figure 3.10).

In contrast, healthy individuals showed a mild negative correlation (r = –0.1863), which approached but did not reach statistical significance (p = 0.0649). Similarly, wild-type PTC patients exhibited a weak negative trend (r = –0.05531, p = 0.6397), while mutant-type individuals also displayed a low inverse correlation (r = –0.1042, p = 0.6124). However, neither of these associations was statistically significant.



Group	Pearson r	p-value
Healthy	-0.1863	0.0649
Thyroid Disease	-0.2472	0.0136
Wild Type	-0.05531	0.6397
Mutant Type	-0.1042	0.6124

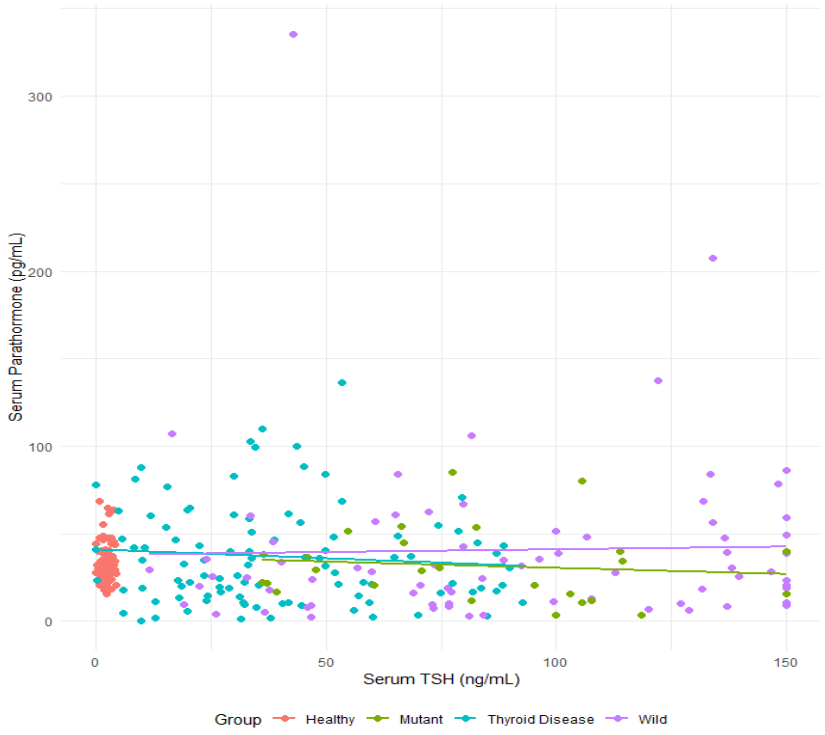
Figure 3.10: Correlation Analysis of Serum TSH and Vitamin D Levels among Study Groups.

These findings suggest that the TSH–vitamin D relationship might be stronger in individuals with benign thyroid disorders compared to those with cancer or healthy conditions.

3.6.4.3 Correlation Analysis between Serum TSH and Parathyroid Hormone Levels across Study Groups

Pearson correlation analysis between serum TSH and parathyroid hormone (PTH) levels showed no statistically significant associations in any of the study groups (Figure 3.11). In healthy individuals, a weak positive correlation was observed (r = 0.09233), but it was not statistically significant (p = 0.3609).

Conversely, individuals with non-tumorous thyroid disease showed a weak negative correlation (r = –0.08919, p = 0.3775), which was not statistically significant. Among papillary thyroid carcinoma (PTC) patients, wild-type individuals exhibited a negligible positive trend (r = 0.02783, p = 0.8139), while mutant-type individuals displayed a slight negative correlation (r = –0.1128, p = 0.5834).



Group	Pearson r	p-value
Healthy	0.09233	0.3609
Thyroid Disease	-0.08919	0.3775
Wild Type	0.02783	0.8139
Mutant Type	-0.1128	0.5834

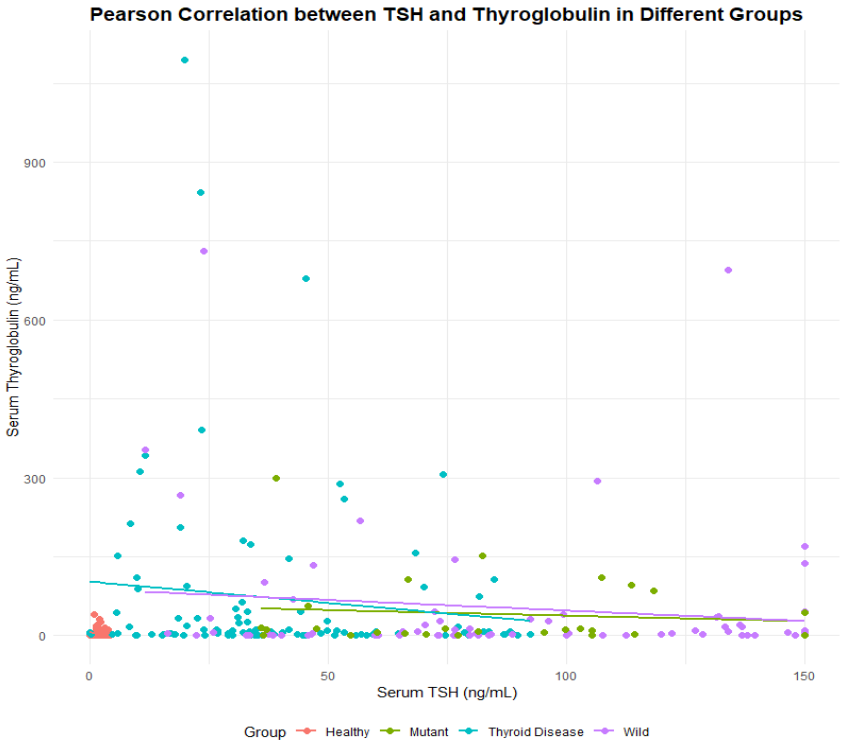
Figure 3.11: Correlation Analysis of Serum TSH and Parathyroid Hormone Levels among Study Groups

Together, these findings indicate that TSH levels are not significantly linked to parathyroid hormone levels in either clinical or healthy groups.

3.6.4.4 Correlation Analysis between Serum TSH and Thyroglobulin Levels across Study Groups

Pearson correlation analysis of serum thyroid-stimulating hormone (TSH) and thyroglobulin (Tg) levels across study subgroups showed no statistically significant relationships (Figure 3.12). In the healthy control group, the correlation was weak and negative (r = –0.05726, p = 0.5715), suggesting little to no interaction between TSH and Tg in individuals with normal thyroid function.

Similarly, participants with non-neoplastic thyroid disorders showed a weak inverse correlation (r = –0.1201), which was not statistically significant (p = 0.2339). Among papillary thyroid carcinoma (PTC) patients, subgroup analyses based on mutational status indicated that both wild-type (r = –0.1319, p = 0.2625) and mutant-type individuals (r = –0.1044, p = 0.6117) exhibited weak, non-significant negative correlations between TSH and Tg levels.



Group	Pearson r	p-value
Healthy	-0.05726	0.5715
Thyroid Disease	-0.1201	0.2339
Wild Type	-0.1319	0.2625
Mutant Type	-0.1044	0.6117

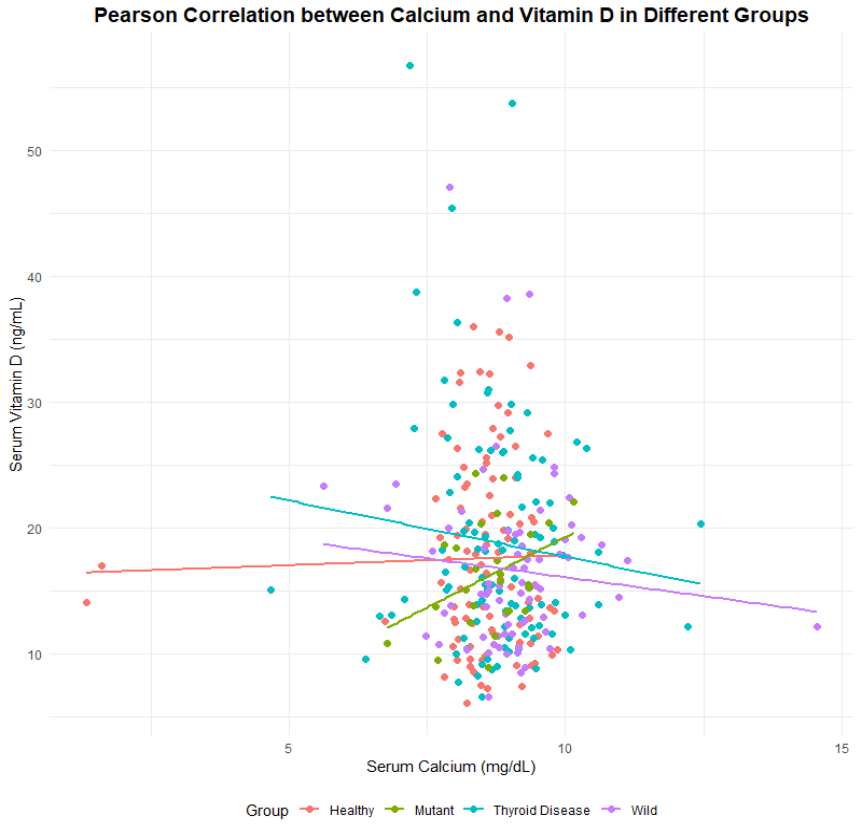
Figure 3.12: Correlation Analysis of Serum TSH and Thyroglobulin Levels among Study Groups.

Collectively, these findings indicate that, within this cohort, fluctuations in serum TSH levels do not significantly affect circulating thyroglobulin levels, regardless of thyroid pathology or

underlying NRAS/RB1 mutational status. This supports the idea that Tg levels are not directly controlled by TSH in either benign or malignant thyroid conditions, especially in the context of altered genomic backgrounds.

3.6.4.5 Correlation Analysis between Serum Calcium and Vitamin D Levels across Study Groups

The Pearson correlation analysis showed different patterns between serum calcium and vitamin D levels across various study groups (Figure 3.13). In the mutant-type PTC group, a statistically significant moderate positive correlation was found (r = 0.3955, p = 0.0455), indicating that individuals with NRAS or RB1 variants experienced a linked increase in calcium as vitamin D levels rose. This may indicate a variant-driven influence on calcium and vitamin D balance.



Group	Pearson r	p-value
Healthy	0.02488	0.8069
Thyroid Disease	-0.1024	0.3134
Wild Type	-0.09886	0.4020
Mutant Type	0.3955	0.0455

Figure 3.13: Correlation Analysis of Serum Calcium and Vitamin D Levels among Study Groups.

These findings reveal a distinctive biochemical profile in mutation-positive individuals, where calcium and vitamin D may be more closely linked, possibly due to altered regulatory mechanisms caused by somatic variants in key signaling genes.

3.6.4.6 Correlation Analysis between Serum Calcium and Parathyroid Hormone Levels across Study Groups

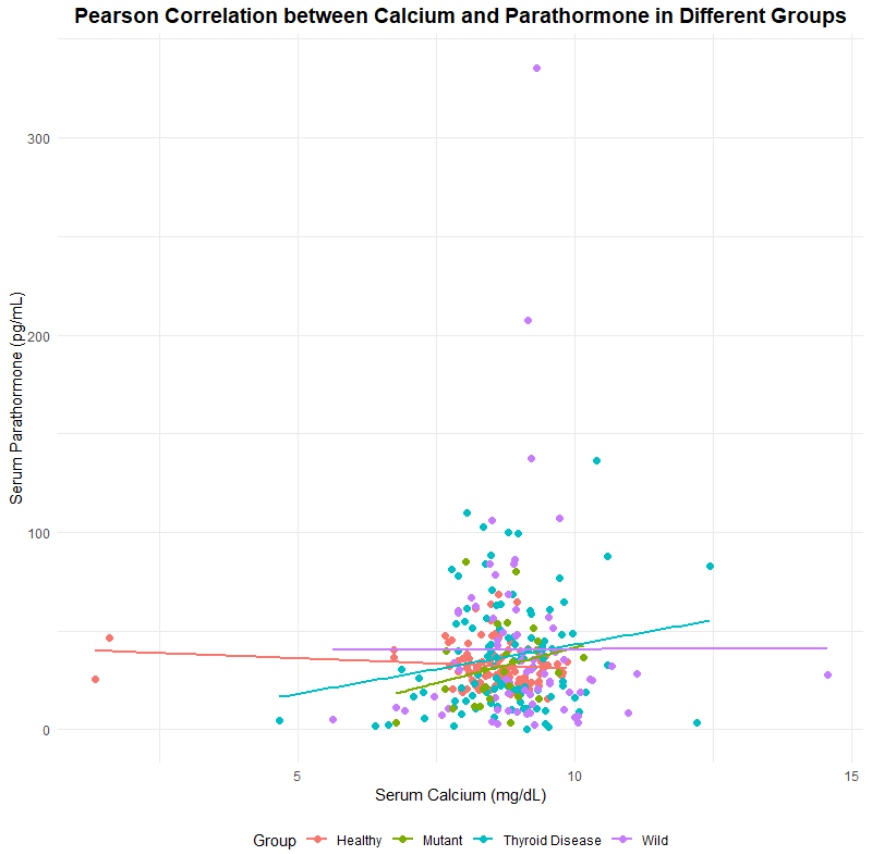
Pearson correlation analysis between serum calcium and parathyroid hormone (PTH) concentrations across all study groups showed no statistically significant relationships. In the mutant-type PTC group, a weak positive correlation was observed ($r = 0.2442$, $p = 0.2292$), indicating a non-significant trend where higher PTH levels might be linked to elevated serum calcium (Figure 3.14). This could suggest subtle changes in calcium regulation, although the result was not statistically significant.

Among wild-type PTC individuals, no significant correlation was found, with a nearly zero coefficient ($r = 0.0023$, $p = 0.9842$), indicating complete independence between calcium and PTH levels in this group.

In the non-tumorous thyroid disease group, a weak positive trend was observed ($r = 0.1842$, $p = 0.0666$), which approached but did not reach the threshold for statistical significance, possibly indicating early or subclinical compensatory regulation of calcium via parathyroid hormone.

Conversely, the healthy control group showed a weak, non-significant inverse correlation ($r = -0.1148$, $p = 0.2552$), aligning with the physiological feedback loop where increased calcium levels inhibit PTH secretion. However, the lack of significance suggests that within normal ranges, these fluctuations may not be strong enough to establish a clear relationship.

Overall, these findings indicate no definitive correlation between calcium and PTH in any group, although slight group-specific trends might need more study in larger groups.



Group	Pearson r	p-value
Healthy	-0.1148	0.2552
Thyroid Disease	0.1842	0.0666
Wild Type	0.002344	0.9842
Mutant Type	0.2442	0.2292

Figure 3.14: Correlation Analysis of Serum Calcium and Parathyroid Hormone Levels among Study Groups.

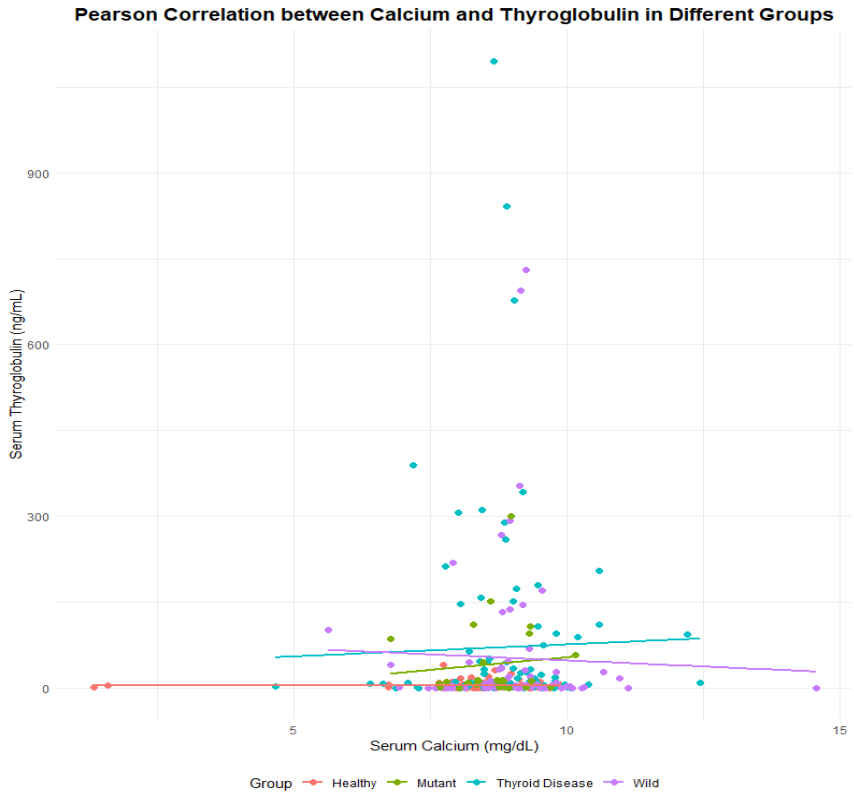
3.6.4.7 Correlation Analysis between Serum Calcium and Thyroglobulin Levels across Study Groups

Pearson correlation analysis revealed no statistically significant link between serum calcium and thyroglobulin (Tg) levels in any of the study groups, indicating an overall lack of a linear relationship between these two variables across various clinical settings (Figure 3.15).

In the mutant-type PTC group, a weak positive correlation was observed ($r = 0.09281$, $p = 0.652$), suggesting a slight tendency for higher thyroglobulin levels to coincide with increased calcium levels. However, the association was not statistically significant, indicating that calcium and Tg levels in mutation-positive individuals are largely independent.

Among wild-type PTC patients, the analysis revealed a similarly weak negative correlation (r = –0.03609, p = 0.7601), further confirming that there is no meaningful association between the variables in this group.

The non-tumorous thyroid disease group showed a very weak positive correlation (r = 0.02607, p = 0.7968), while the healthy control group exhibited an almost null correlation (r = –0.006038, p = 0.9525), neither of which has statistical or biological significance.



Group	Pearson r	p-value
Healthy	-0.006038	0.9525
Thyroid Disease	0.02607	0.7968
Wild Type	-0.03609	0.7601
Mutant Type	0.09281	0.652

Figure 3.15: Correlation Analysis of Serum Calcium and Thyroglobulin Levels among Study Groups.

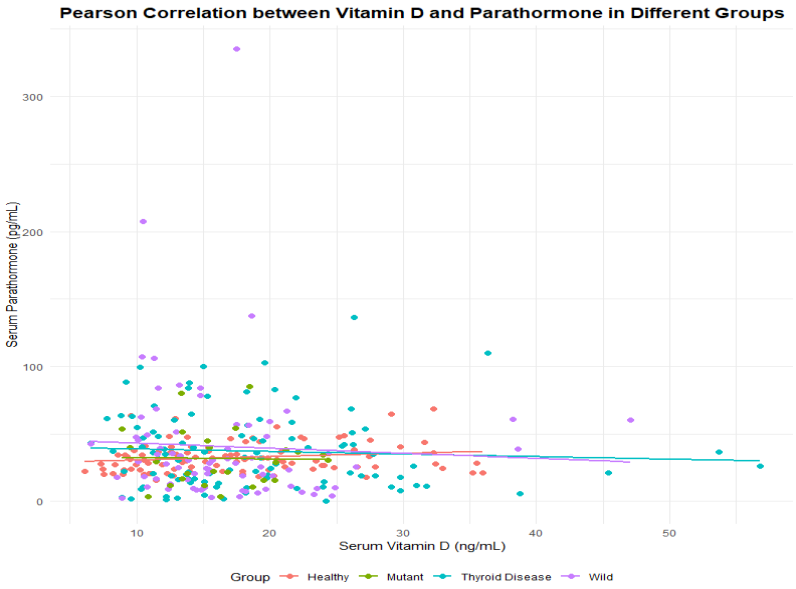
Overall, these findings indicate that thyroglobulin levels do not have a significant correlation with serum calcium in healthy individuals, thyroid disease patients, or PTC subgroups, suggesting that these parameters are likely regulated independently within the physiological and pathological contexts examined in this study.

3.6.4.8 Correlation Analysis between Serum Vitamin D and Parathyroid Hormone Levels across Study Groups

Pearson correlation analysis between vitamin D and parathyroid hormone (PTH) levels showed no statistically significant relationships in any of the study groups, indicating there is no meaningful linear association between these two variables in the populations studied (Figure 3.16).

In the healthy control group, there was a weak positive correlation (r = 0.1695, p = 0.0934), indicating a slight tendency for higher vitamin D levels to be associated with increased PTH levels. However, this correlation was not statistically significant, suggesting that vitamin D and PTH levels in healthy individuals are mostly independent.

Among individuals with thyroid disease, the analysis showed a very weak negative correlation (r = –0.06104, p = 0.5484), further supporting the lack of a significant relationship between the two variables in this group. For the wild-type group, the results indicated a similarly weak negative correlation (r = –0.0525, p = 0.6569), confirming that vitamin D and PTH levels do not have a meaningful association in this population.



Group	Pearson r	p-value
Healthy	0.1695	0.0934
Thyroid Disease	-0.06104	0.5484
Wild Type	-0.0525	0.6569
Mutant Type	-0.02136	0.9175

Figure 3.16: Correlation Analysis of Serum Vitamin D and Parathyroid Hormone Levels among Study Groups.

The mutant type group demonstrated an extremely weak negative correlation (r = –0.02136, p = 0.9175), which was far from statistically significant, reinforcing the conclusion that there is no notable relationship between vitamin D and PTH levels in this group either.

In summary, the findings indicate that vitamin D levels do not have a significant correlation with parathyroid hormone levels among healthy individuals, thyroid disease patients, or the mutant and wild-type subgroups. This suggests that vitamin D and PTH are regulated independently across the studied physiological and pathological conditions.

3.6.4.9 Correlation Analysis between Serum Vitamin D and Thyroglobulin Levels across Study Groups

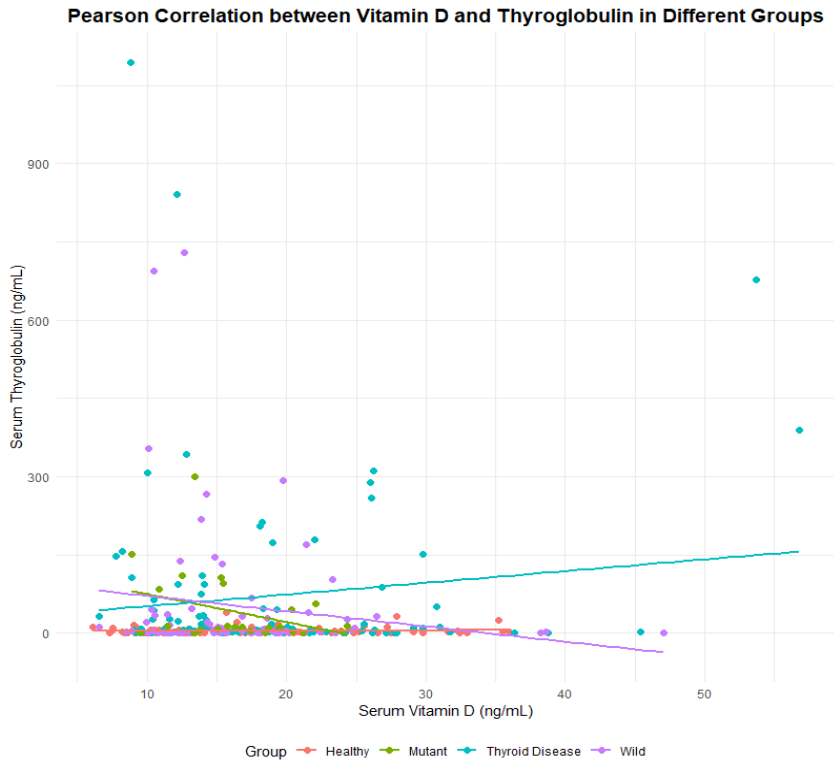
Pearson correlation analysis between serum vitamin D and thyroglobulin (Tg) levels showed variable associations across study groups, with statistical significance only in the mutant-type group (Figure 3.17).

In healthy individuals, a very weak positive correlation was observed (r = 0.0592, p = 0.5605), indicating no significant linear relationship between vitamin D and thyroglobulin levels in this population.

Among patients with non-neoplastic thyroid diseases, a similarly weak positive correlation was observed (r = 0.1212, p = 0.2319), which was also statistically nonsignificant, indicating independent variation of these biomarkers in thyroid dysfunction without malignancy.

In contrast, the wild-type PTC subgroup showed a weak negative correlation (r = –0.151, p = 0.199), which did not reach statistical significance but suggested a possible inverse trend between vitamin D and Tg in this group.

Interestingly, in the mutant-type PTC subgroup, a moderate and statistically significant negative correlation was observed (r = –0.3918, p = 0.0477), indicating that lower vitamin D levels may be linked to higher thyroglobulin concentrations in individuals with specific genetic mutations.



Group	Pearson r	p-value
Healthy	0.0592	0.5605
Thyroid Disease	0.1212	0.2319
Wild Type	-0.151	0.199
Mutant Type	-0.3918	0.0477

Figure 3.17: Correlation Analysis of Serum Vitamin D and Thyroglobulin Levels among Study Groups.

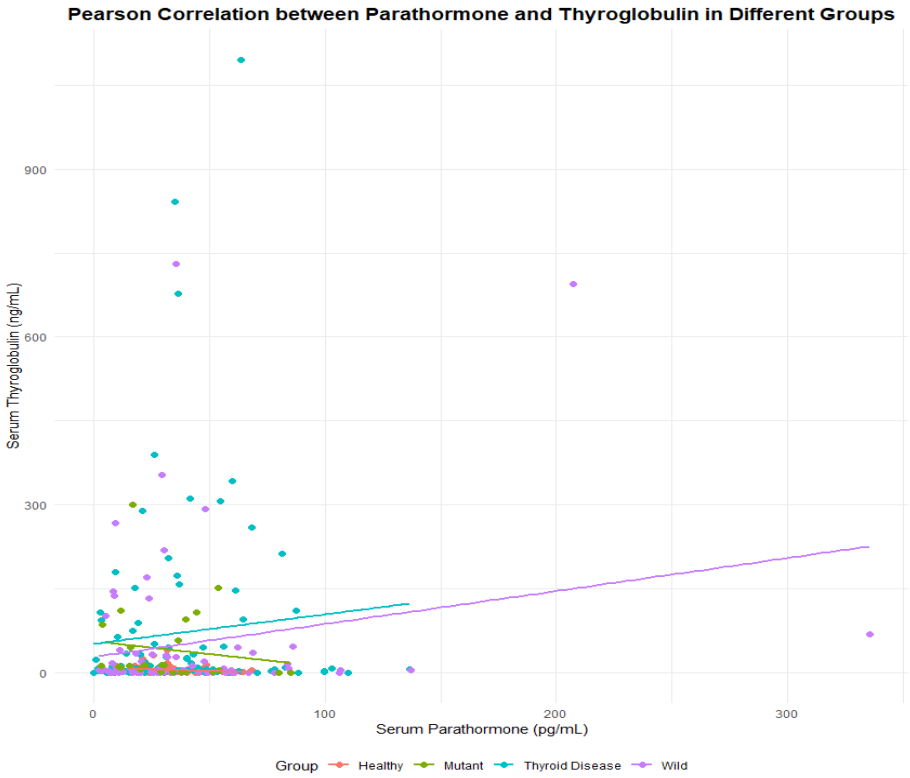
Overall, these results suggest that while vitamin D and thyroglobulin levels are mostly unrelated in healthy individuals and those with non-malignant thyroid conditions, there may be a genotype-dependent link in PTC, especially in mutation-positive cases. This finding calls for further research to explore possible mechanistic connections between vitamin D metabolism and tumor marker expression in genetically vulnerable thyroid cancer patients.

3.6.4.10 Correlation Analysis between Serum Parathyroid Hormone and Thyroglobulin Levels across Study Groups

Pearson correlation analysis between serum parathyroid hormone (PTH) and thyroglobulin (Tg) levels showed no statistically significant relationships across any study groups, indicating a general lack of linear association between these two parameters (Figure 3.18).

In the healthy control group, a weak negative correlation was observed (r = –0.1704, p = 0.0902), indicating a slight inverse trend between PTH and Tg levels. However, the association did not reach statistical significance.

Among patients with non-malignant thyroid diseases, the analysis showed a weak positive correlation (r = 0.08829, p = 0.3824), indicating no significant relationship between PTH and Tg levels in this group.



Group	Pearson r	p-value
Healthy	-0.1704	0.0902
Thyroid Disease	0.08829	0.3824
Wild Type	0.2182	0.0618
Mutant Type	-0.1404	0.494

Figure 3.18: Correlation Analysis of Serum Parathyroid Hormone and Thyroglobulin Levels among Study Groups.

In the wild-type PTC subgroup, a moderate positive correlation was observed (r = 0.2182, p = 0.0618), nearing statistical significance. This trend may indicate a potential biological interaction between PTH regulation and thyroglobulin production in genetically unaltered PTC cases, although further validation is needed.

Meanwhile, the mutant-type PTC group showed a weak negative correlation ($r = –0.1404$, $p = 0.494$), which was not statistically significant, suggesting independent variation of PTH and Tg in mutation-positive individuals.

Overall, these findings indicate that serum PTH and thyroglobulin levels do not show a consistent or statistically significant correlation across healthy individuals, patients with thyroid diseases, or PTC subgroups. The moderate trend observed in the wild-type group may warrant further investigation to examine potential genotype-specific endocrine interactions.

Chapter 4

Discussion

Papillary thyroid carcinoma (PTC) is the most common subtype of thyroid cancer, usually showing slow growth but capable of presenting with diverse clinical and molecular features. This study presents an integrated molecular and biochemical analysis of PTC in a Bangladeshi population, encompassing next-generation sequencing (NGS) of cancer-related genes, targeted Sanger sequencing of key loci, and serum biomarker profiling. By linking molecular genetics with biochemical and clinical data, this research reveals potential mechanisms underlying tumor diversity and highlights mutation-related metabolic disruptions, particularly those affecting calcium balance.

Genetic Landscape of PTC in the Bangladeshi Population

The targeted NGS with the Ion AmpliSeq™ Cancer Hotspot Panel v2 identified a broad spectrum of somatic and germline variants across various oncogenic pathways in both blood and FFPE-derived tissue DNA. Interestingly, blood samples showed a higher mutational burden than the corresponding tumor tissues in most cases (Figure 3.1). This difference likely results from tumor heterogeneity, in which genetic mutations from subclonal populations may not be fully detected in formalin-fixed paraffin-embedded (FFPE) tissue samples. Similar results have been reported in recent studies, emphasizing that blood-based sequencing can detect a higher mutational burden due to circulating tumor DNA (ctDNA) from tumor subclones, whereas formalin-fixed tissues may overlook these subclonal variants (Xie et al., 2023; Gilson et al., 2022).

Among the genes involved in tissue samples were well-known thyroid drivers—BRAF, HRAS, NRAS, MET, and RET—along with classic tumor suppressors such as RB1 and TP53 (Table 3.6). Importantly, the mutations identified were located in critical functional regions: exon 15 of BRAF (V600E), exon 3 of NRAS (Q61K), and exon 20 of RB1 (I680T) (Table 3.7). These findings align with the current consensus in the literature, which indicates that the BRAF V600E mutation is a key driver of MAPK pathway activation in approximately 30% to 83% of cases of papillary thyroid carcinoma (PTC) worldwide. Notably, this mutation is significantly more prevalent among Asian populations, with a prevalence of approximately 75% in The Cancer Genome Atlas (TCGA) data (Prete et al., 2021). Similarly, NRAS/HRAS codon 61 mutations are recognized contributors to follicular-pattern thyroid tumors and are less frequently associated with classic PTC (Jin M et al., 2021). The significance of RB1 mutations is relatively recent; although less studied, emerging

evidence suggests that the loss of its tumor-suppressive function may facilitate the development of aggressive PTC variants (Iványi et al., 2025; Rajabi et al., 2022). The TP53 Arg72Pro polymorphism has been previously linked to thyroid and other solid tumors in Asian populations (Lacka et al., 2025); however, findings vary across different regions. In a meta-analysis by Fischer et al. (2023), the Pro/Pro genotype was associated with a slightly increased risk of thyroid cancer under a recessive model, particularly in non-European groups.

A notable aspect of this study was the high prevalence of specific synonymous and intronic germline variants, such as FGFR 3 p. Thr 651 =, PDGFRA p. Pro 567 =, KDR c. 798 + 54 G>A, and APC p. Thr 1493 =, detected in the blood- derived DNA of PTC patients (Table 3.4. 4). These findings are consistent with those of Mio et al. (2021), who reported that rare germline variants, particularly in genes associated with DNA repair, could increase susceptibility to papillary thyroid carcinoma. Although these variants are functionally silent at the protein level or located in non-coding regions, they may act as regulatory modulators, splicing modifiers, or expression-altering polymorphisms. The FGFR3 p. Thr 651 = variant is located within a conserved region and has been previously associated with alterations in transcriptional stability and splicing in epithelial tumors such as bladder and skin cancers (Pace et al., 2023; Lafitte et al., 2013). The PDGFRA p. Pro 567 = variant has been identified in gastrointestinal stromal tumors and gliomas, with recent studies suggesting that synonymous mutations in PDGFRA can influence translational efficiency and downstream signaling, potentially affecting tumor progression (Lopez-Campistrous et al., 2021; Shi et al., 2020). The KDR c. 798 + 54 G>A intronic variant, although not extensively studied in thyroid cancer, is located within a gene critical to angiogenesis. Intronic mutations in KDR have been linked to abnormal splicing and disrupted angiogenesis in other cancers, and KDR overexpression has been observed in aggressive PTC subtypes (Wu et al., 2024; Butt et al., 2019). The APC p. Thr 1493 = synonymous variant occurs in a mutation-prone region of the APC gene, which is involved in Wnt signaling. Synonymous APC variants are thought to affect exon splicing or mRNA secondary structure, potentially impacting tumor suppressor functions. Although APC is traditionally associated with colorectal cancer, abnormal methylation and mutations have also been reported in thyroid and endocrine tumors (Tufail et al., 2025). Their high prevalence indicates a shared polymorphic background within the Bangladeshi population that might contribute as low-penetrance modifiers in thyroid tumor development.

Allele frequency comparisons showed stark differences between the studied cohort and global/Asian populations (Table 3.5). Some variants, such as CSF1R rs386693509, were absent from public databases but were present at significant frequencies in this cohort, suggesting possible population-specific or founder mutations. The presence of these variants in the Bangladeshi PTC cohort suggests a potentially distinct regional genomic background that's underrepresented in major global cancer variant databases, such as TCGA, gnomAD, and 1000 Genomes. These findings support the creation of region-specific variant interpretation databases and genetic screening panels to improve diagnostic accuracy and prevent misclassifying benign variants as pathogenic.

Identification and Characterization of Novel Variants in PTC

In this study, twelve somatic mutations were validated using Sanger sequencing across key oncogenic drivers—HRAS, NRAS, RET, and RB1—in a cohort of 100 patients with papillary thyroid carcinoma (Table 3.10). Among these, three novel variants—NRAS c.7775T>A, NRAS c.7797G>A, and RB1 c.161020T>A—were identified in regulatory intronic and exonic regions (Table 3.11). Notably, the NRAS c.7797G>A, an intronic variant, was identified in 18% of the mutant group and was not present in wild-type individuals, showing a statistically significant link to mutation-positive status ($p < 0.0001$) (Table 3.14). While NRAS codon 61 mutations are well-known in thyroid neoplasms, intronic NRAS variants are less reported, making this a novel regulatory candidate for PTC susceptibility (Shi et al., 2024).

Similarly, the RB1 c.161020T>A variant, located in exon 20, showed a strong statistical link to PTC cases ($p < 0.0001$) (Table 3.14). Although RB1 mutations are rarely studied in thyroid cancer, recent research has emphasized RB1's role in cell cycle arrest and chromatin remodeling, with implications for endocrine tumors, including PTC (Shi et al., 2020). The predicted pathogenicity of RB1 p.Ile680Asn supports its possible role in tumor progression.

In contrast, HRAS synonymous variants, such as c.6309T>C, and upstream transcript variants, like c.6219C>T, were detected but did not show a significant association with disease status, indicating a limited functional impact (Table 3.11).

These findings differ from large-scale datasets such as TCGA, which primarily report BRAF, RAS, and RET mutations but do not include intronic NRAS or non-hotspot RB1 variants (Chauhan et al., 2025; Uno et al., 2024). The absence of these variants in previous genomic studies indicates a population-specific mutational profile, possibly influenced by ethnic or environmental factors. Due to their exclusivity and predicted functional importance, NRAS c.7797G>A and RB1 p.Ile680Asn warrant further research as potential biomarkers for PTC development.

Mutation-Phenotype Associations and Risk Stratification

In our cohort, NRAS and RB1 mutations were associated with earlier disease onset, particularly among patients aged 40 or older, suggesting a possible acceleration of tumor development associated with these genetic changes (Table 3.13). These variations were notably more common in intermediate-risk PTC cases, absent in the low-risk group, and only slightly present in high-risk patients, suggesting they may play a role in disease progression rather than extreme aggressiveness (Table 3.21).

This observation aligns with recent findings by Pozdeyev et al. (2018), who reported that non-hotspot mutations in RB1 and RAS genes were more common in advanced differentiated thyroid cancers, but not necessarily in the most aggressive anaplastic subtypes. Similarly, a 2025 case report by Chauhan et al. described a PTC with multiple driver mutations, including NRAS Q61H and PTEN. Yet, it showed non-aggressive clinical behavior, reinforcing the idea that mutation burden alone may not determine tumor severity.

Furthermore, Fu et al. (2024) showed that RAS variants, including NRAS, are primarily associated with low- to intermediate-risk follicular-patterned thyroid cancers and rarely co-occur with high-risk markers such as TERT promoter mutations. This supports our view that NRAS and RB1 variants may play a role in early oncogenic events or progression, but are not definitive signs of poor prognosis.

Overall, these comparisons suggest that NRAS and RB1 mutations may serve as molecular markers of intermediate-risk PTC, potentially assisting in risk assessment and personalized monitoring strategies, especially in patients with early-onset disease.

Stratified analysis of mutation distribution revealed important demographic and clinical patterns. Female patients made up the majority of mutation-positive cases (21 out of 26), consistent with the well-known higher incidence of thyroid cancer in women (Table 3.15). A recent study by Valenzano et al. (2025) highlighted sex-based differences in thyroid nodule morphology and oncogenic risk, attributing the difference to estrogen receptor signaling and immune modulation in female patients.

Interestingly, the NRAS c.7797G>A intronic variant showed significant enrichment among male participants ($p = 0.0038$), indicating that its penetrance may be independent of sex hormone–mediated pathways (Table 3.15). This finding aligns with results from Fu et al. (2024), who reported variability in RAS mutations across sexes, with no consistent association with hormonal status.

Smoking status revealed notable trends. Although only a small number of patients were smokers, NRAS and RB1 variants were strongly associated with non-smoking individuals ($p < 0.0001$), indicating a primarily sporadic mutational origin rather than environmentally triggered carcinogenesis (Table 3.16). This finding aligns with data from the Cancer Genome Atlas (TCGA), which highlights thyroid cancer's low mutational burden and limited exposure to external carcinogens (Landa et al., 2024).

Family history analysis further supports the hypothesis of sporadic mutations. The strongest associations for NRAS and RB1 variants were observed in patients without a family history of thyroid or other cancers (Table 3.18). Although a few familial cases carried these variants, their low frequency and lack of statistical significance emphasize the dominance of sporadic mutagenesis in this group. These results are consistent with those of Bakht et al. (2024), who showed that HRAS/NRAS mutations in indeterminate thyroid nodules are more common in sporadic cases and are rarely associated with familial clustering.

Clinical and Staging Associations of NRAS and RB1 Variants

Patients carrying at least one of the three evaluated variants—NRAS 7775T>A, NRAS 7797G>A, and RB1 161020T>A—collectively referred to as the mutant-type group, showed a heterogeneous distribution across TNM classifications and clinical risk categories (Table 3.20). A key finding

was the significant enrichment of the NRAS 7797A allele in small, localized tumors (T1), suggesting its involvement in early tumor development. This supports previous findings that RAS mutations often happen during early follicular cell transformation and are commonly associated with encapsulated or minimally invasive papillary thyroid carcinoma (PTC) phenotypes (Hernandez-Prera et al., 2024; Brandler et al., 2023; Jung et al., 2021).

Conversely, the RB1 161020A variant was detected in tumors across intermediate- to advanced-stage tumors, suggesting a broader role in disease progression. While RB1 is traditionally recognized as a tumor suppressor with essential functions in cell cycle regulation, its involvement in thyroid cancer has only recently gained attention. Emerging evidence suggests that loss-of-function RB1 mutations may promote cellular dedifferentiation and proliferative changes within thyroid neoplasms (Huang et al., 2024; Leandro-García et al., 2023).

Risk stratification showed a high prevalence of these variants in intermediate-risk patients, with lower frequencies in the high- and low-risk groups (Table 3.21). This pattern of enrichment may define a unique molecular subset of PTC, marked by intermediate clinical behavior. A similar trend was observed in a European cohort, in which RAS-driven PTC cases generally did not exhibit either pronounced indolence or a clearly aggressive course (Sang et al., 2024; Jeong et al., 2021).

Interestingly, the occurrence of NRAS and RB1 mutations in high-risk and metastatic PTC cases was relatively low. Although this distribution may seem paradoxical—considering the known link between mutations and oncogenic potential—it supports the idea that these genetic changes may mainly act as early triggers rather than direct causes of metastatic progression. This view is consistent with stratification models from the Cancer Genome Atlas, which differentiate RAS-mutant tumors from BRAF V600E-driven, high-grade PTC subtypes (Kim et al., 2024; Bonaldi et al., 2021).

It must be acknowledged, however, that the low number of mutations observed in advanced-stage tumors may be due to technical limitations of the targeted sequencing method. Future studies with broader panels or whole-exome/transcriptomic sequencing may identify additional driver mutations or synergistic events that drive high-grade transformation and metastatic behavior.

Collectively, the distribution of NRAS and RB1 variants in this cohort supports their suspected role in tumor initiation and early progression, with limited presence in advanced disease. These

findings support integrating mutational profiling into clinical risk models, particularly in intermediate-risk cases, where additional molecular insights can inform treatment decisions.

Biochemical Profiles and Their Clinical Implications

Comparative biochemical profiling across three groups, termed healthy individuals, patients with benign thyroid conditions, and those with papillary thyroid carcinoma (PTC), revealed distinct metabolic signatures (Table 3.22). Thyroid-stimulating hormone (TSH) levels were significantly higher in PTC patients, averaging 86.81 ± 4.08 ng/mL, compared to non-neoplastic thyroid disease patients (39.33 ± 2.49 ng/mL) and healthy controls (2.167 ± 0.107 ng/mL). This elevation supports the role of TSH as a mitogenic driver of thyroid follicular cells, with emerging evidence linking elevated serum TSH levels to tumor progression and poorer prognosis in differentiated thyroid cancers (Xu et al., 2023; Kim et al., 2022; Huang et al., 2017). Notably, Huang et al. (2017) identified sex-specific links between abnormal TSH levels and higher PTC risk, indicating a complex endocrine influence on tumor biology.

Serum calcium levels were lower in patients with thyroid cancer than in healthy individuals, potentially indicating tumor-related disruption of calcium homeostasis, parathyroid dysfunction, or changes in vitamin D metabolism (Table 3.22). This hypocalcemia was more severe in mutation-positive individuals carrying NRAS or RB1 variants (8.514 ± 0.1621 mg/dL) compared to their wild-type counterparts (9.042 ± 0.1304 mg/dL), suggesting that these variants may play a role in calcium signaling imbalance (Table 3.23). Recent transcriptomic analyses by Hu et al. (2023) identified gene signatures associated with aggressive PTC phenotypes, including changes in the expression of calcium transporters, CAMK signaling components, and voltage-gated calcium channels.

These findings emphasize the connection between endocrine and ionic regulators in PTC development, indicating that TSH elevation and calcium imbalance may act as biochemical markers of tumor aggressiveness, especially in individuals with a genetic predisposition.

Correlation between Biochemical Profiles and Genotypic Variants in PTC

Correlation analyses uncovered subtle links between biochemical markers and genotypic status in papillary thyroid carcinoma (PTC). Across the entire cohort, no significant linear relationships were observed between serum calcium and other parameters such as vitamin D, parathyroid hormone (PTH), or thyroglobulin (Tg), suggesting a complex regulatory system beyond straightforward biochemical connections.

However, in mutation-positive individuals, a moderate positive correlation between calcium and vitamin D was observed ($r = 0.3955$, $p = 0.0455$), possibly indicating compensatory endocrine regulation in the presence of NRAS or RB1 variants (Figure 3.13). In contrast, the wild-type group showed a non-significant inverse trend, indicating possible genotype-dependent differences in calcium–vitamin D homeostasis. A statistically significant negative correlation was again observed between TSH and calcium levels in the mutant group ($r = –0.3989$, $p = 0.0435$), indicating a potential disruption in calcium metabolism in mutation-positive PTC patients (Figure 3.9). This finding agrees with recent evidence from Hu et al. (2023), who showed that calcium metabolism–related gene signatures can predict poor outcomes in PTC and are associated with immune dysregulation and changes in the tumor microenvironment.

Furthermore, dysregulation of the vitamin D axis has been linked to thyroid tumor development, with emerging evidence suggesting its role in genomic instability and faulty differentiation. A comprehensive review by Maciejewski & Lacka (2022) highlighted the impact of vitamin D-related gene variants (e.g., VDR, CYP24A1, DHCR7) on thyroid cancer risk and progression.

Regarding thyroglobulin (Tg), no significant correlations were observed with other serum markers, nor were there notable differences between wild-type and mutant groups. This may reflect heterogeneous Tg expression in well-differentiated tumors and the potential confounding effect of anti-Tg antibodies, which were not assessed in this study. Prior studies have emphasized that Tg levels alone may not reliably predict recurrence unless interpreted in the context of antibody status and imaging findings (Zahra et al., 2021; Yang et al., 2015).

Limitations of the Study

Despite the comprehensive molecular and biochemical analysis in this study, some limitations should be acknowledged when applying the results:

Sample Size and Population Scope

While a sufficient number of participants (n = 100) allows for the identification of statistically significant relationships, the sample size remains relatively small compared to other multicenter genomic studies. Recruiting primarily Bangladeshi participants from a single center requires caution when applying these findings to all South Asians or broader populations. Larger, multi-ethnic studies are crucial for verifying the prevalence and health impacts of these variants.

Targeted Gene Panel Constraints

Mutation screening was limited to predefined hotspots in specific oncogenes and tumor suppressor genes using the Ion AmpliSeq™ Cancer Hotspot Panel v2. While this approach was cost-effective, it did not encompass other potentially relevant genomic regions. As a result, potentially non-hotspot driver mutations and structural variants (such as gene fusions and copy number alterations) may have been missed. Whole-exome or whole-genome sequencing would likely provide a more comprehensive view of the mutation spectrum.

Functional Validation of Variants

The newly identified novel variants in NRAS (c.7775T>A, c.7797G>A) and RB1 (c.161020T>A; p.Ile680Asn) showed strong statistical associations with PTC. However, these have not been confirmed through in vitro or in vivo functional assays, such as splicing tests, reporter gene systems, or CRISPR-mediated knock-in models. Additionally, because there is no direct functional data, the causative role of these variations in PTC development remains only speculative.

Biochemical Assay Constraints

While biochemical profiling showed significant correlations (e.g., hypocalcemia in mutation-positive subjects), serum data were limited to baseline measurements and were not available for longitudinal analysis. Monitoring TSH, calcium, vitamin D, PTH, and thyroglobulin over time, especially before and after treatment, could provide more detailed genotype-related metabolic

insights. Additionally, anti-thyroglobulin antibody (TgAb) status was not evaluated, which might have impacted the interpretation of thyroglobulin levels.

Clinical Stratification Limitations

Risk group classification was also performed following American Thyroid Association (ATA) guidelines; however, the number of patients in the high- and low-risk groups was small in this sample, which may indicate insufficient statistical power for subgroup analysis. Therefore, conclusions about the enrichment of variants in intermediate-risk categories should be interpreted with caution and confirmed in larger cohorts with proportional representation of subgroups.

Environmental and Epigenetic Factors Not Evaluated

Although the present study focused on somatic mutations, we did not analyze detailed environmental exposures (e.g., iodine intake, radiation history, dietary factors) or epigenetic modifications (e.g., DNA methylation, histone marks). These unmeasured factors could have affected the associations between mutation and biochemistry.

Cross-Sectional Study Design

This study is cross-sectional and cannot determine causality among genetic variants, biochemical dysregulation, and clinical outcomes. A longer follow-up is necessary to evaluate the significance of these mutations in prognosis, as well as their effects on recurrence, metastasis, and therapy response.

Conclusion

This research provides a comprehensive molecular and biochemical analysis of papillary thyroid carcinoma (PTC) in a Bangladeshi population, using targeted next-generation sequencing (NGS), Sanger sequencing validation, and serum biomarker profiling. The study identifies and characterizes two novel intronic variants in NRAS (g.7775T>A and g.7797G>A) and a missense variant in RB1 (c.2039T>A; p.Ile680Asn) that are significantly associated with mutation-positive PTC cases. These variants are common among individuals without a family history of thyroid disease and among non-smokers. These findings strongly suggest a sporadic pattern of tumor

development, possibly influenced by region-specific environmental or epigenetic factors, rather than inherited or exposure-related carcinogenesis.

Clinically, these mutations were more common among patients classified as intermediate risk and those with tumors of small to moderate size. Instead of indicating a highly aggressive disease, these changes seem to contribute to early tumor development and may identify a distinct molecular subtype of PTC. This stratification has implications for improving diagnostic and prognostic methods, particularly given region-specific disease patterns.

From a biochemical perspective, genotype-specific changes in systemic metabolic markers were observed. Mutation-positive patients exhibited lower serum calcium, distinct vitamin D-thyroglobulin relationships, and elevated TSH levels. These results suggest that NRAS and RB1 mutations may disrupt endocrine–genetic axes, particularly in calcium homeostasis and thyroid–parathyroid signaling. The positive correlation between vitamin D and calcium in mutation carriers, along with a unique inverse relationship between vitamin D and thyroglobulin, emphasizes the potential for these mutations to influence tumor biology through metabolic pathways beyond conventional oncogenic signaling.

These findings support a more integrated model of thyroid cancer biology, in which somatic mutations in NRAS and RB1 influence not only cellular proliferation pathways (e.g., MAPK, PI3K–Akt) but also metabolic and endocrine regulatory systems. This approach may ultimately enable more precise molecular and metabolic subtyping of PTC and guide personalized treatment strategies.

By establishing NRAS and RB1 variants as candidate biomarkers for early detection and risk stratification, this study paves the way for integrating mutation-guided decision-making into standard clinical practices. The inclusion of serum calcium and vitamin D markers, alongside genotypic data, offers a promising method for metabolically informed patient stratification, particularly in resource-limited settings such as Bangladesh and other South Asian regions. Future research should focus on validating the functions of these new variants, investigating their downstream transcriptional and signaling effects, and evaluating their potential for use in clinical risk-stratification tools. Furthermore, developing region-specific genetic screening panels and metabolically informed diagnostic algorithms will improve the delivery of personalized care for PTC patients.

In conclusion, this study emphasizes the importance of integrating genetic mutation analysis with biochemical profiling to improve our understanding, classification, and management of papillary thyroid carcinoma. It supports the expanding recognition that both genomic and metabolic changes are crucial to thyroid cancer development and progression, offering a promising route for more precise and personalized treatment strategies.

Chapter 5

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Chapter 6

Appendices

Questionnaire sample of the study

Department of Biochemistry and Molecular Biology

University of Dhaka

Study Title: Molecular and Biochemical Investigation of Papillary Thyroid Carcinoma in the Bangladeshi Population

Section A: Participant Identification & Consent

1. Participant ID (Assigned code): _____
2. Date of Interview: _____
3. Interviewer Name & Signature: _____

Section B: Demographic Information

1. Age (years): _____
2. Sex: □ Male □ Female □ Other
3. Ethnicity: □ Bengali □ Other (specify) _____
4. Place of residence: □ Urban □ Rural □ Semi-urban

Section C: Lifestyle & Environmental Exposure

1. Smoking status: □ Current □ Former □ Never
    - If yes, duration (years): _____ Quantity/day: _____
2. Betel nut or tobacco chewing: □ Yes □ No
3. Alcohol consumption: □ Yes □ No
4. Occupational exposure to radiation/chemicals: □ Yes □ No (specify) _____
5. Residential proximity to industrial areas: □ Yes □ No

Section D: Family History

1. Family history of thyroid cancer: □ Yes □ No
    - If yes, relation: _____
2. Family history of other cancers: □ Yes □ No
    - If yes, specify: _____
3. Family history of benign thyroid disease: □ Yes □ No

Section E: Clinical Presentation

1. Duration of symptoms before diagnosis: _____ months
2. Symptoms at presentation:
   □ Neck swelling □ Hoarseness □ Dysphagia □ Pain □ Palpable lymph nodes □ Other (specify) _____
3. Comorbidities:
   □ Diabetes □ Hypertension □ Auto-immune thyroiditis □ Others_____

Section F: Tumor Characteristics & Staging

1. Histopathological type: □ Classical PTC □ Follicular variant □ Other _____
2. Tumor size (cm): _____
3. TNM stage:
   - T: □ T1 □ T2 □ T3 □ T4
   - N: □ N0 □ N1a □ N1b
   - M: □ M0 □ M1
4. Risk category (ATA guidelines): □ Low □ Intermediate □ High

Section G: Research Consent for Future Studies

Do you consent for your stored biological samples and data to be used for future research related to thyroid cancer?
□ Yes □ No

Data Collected by: ……