Oncogenic Alterations and their Implication in Thyroid Cancer

By

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(Avik Chakraborty)

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

(Avik Chakraborty)

List of Publications arising from the thesis:

a. Published:

1. ***BRAF*^{V600E} Mutation in Papillary Thyroid Carcinoma: Significant Association with Node Metastases and Extra Thyroidal Invasion** Avik Chakraborty[1], Archana Narkar[1], Rita Mukhopadhyaya[2], Shubhada Kane[3], Anil D'Cruz[4], M.G.R Rajan[1]. 1. Radiation Medicine Centre, BARC, 2. Molecular Biology Division, BARC 3. Department of Pathology, Tata Memorial Hospital 4. Head and Neck Services, Tata Memorial Hospital; Endocrine Pathology 2012 Jun; 23(2):83-93. DOI 10.1007/s12022-011-9184-5

b. Papers presented in Conferences:

1. **Coexistence of RET/PTC but not PAX8-PPARγ translocation with BRAF mutation in Poorly Differentiated Papillary Thyroid Carcinomas;** A. Chakraborty, R. Mukhopadhyay, S. Kane, M. Rajan*, A. A. Narkar; Abstract No. B221, 7th NCRI International Cancer Conference organized by National Cancer Research Institute in Liverpool, UK. (2011) (available online: http://www.ncri.org.uk)
2. ***BRAF*^{V600E} Mutation in Papillary Thyroid Carcinoma: Significant Association with Node Metastases and Extra Thyroidal Invasion,** Avik Chakraborty, Archana Narkar, Rita Mukhopadhyaya, Shubhada Kane, Anil D'Cruz, M.G. R Rajan; Oral presentation in Bhabha Atomic Research Centre BioScience Meet 2012 (29th Mar, 2012; Session 16:00-16:30)
3. **Prevalence of BRAF and NRAS Mutations in Thyroid Cancer Patients;** Avik Chakraborty, R. Mukhopadhay, S.V. Kane, M.G.R Rajan and A.A. Narkar. Presented at the 5th Annual Conference of Indian Thyroid Society 2008, Chandigarh , 8-9 Feb 2008
4. **Detection of Missense Mutations in B Type Raf Kinase Gene in Thyroid Carcinoma"** Avik Chakraborty, A.A.Narkar, R. Mukhopadhyay, S.V. Kane, MGR

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c. Communicated:

BRAF ^{V600E} Mutation Along with a Rare *BRAF* ^{I582M} Mutation Documented in Tall Cell Variant of Papillary Thyroid Carcinoma with Multiple Neck Recurrences: Case Report. Authors: Avik Chakraborty, MSc, [1, #] Sandip Basu, MBBS(Hons), DRM, DNB, MNAMS, [1, #] S.V Kane, MD,[2] M.G.R Rajan, MSc, PhD, [1, *] Mukund N Sable, MD,[2] ; Journal of Laryngology and Otology.

d. Manuscript under preparation:

Coexistence of RET/PTC and PAX8-PPARγ translocation with *BRAF* mutation in Poorly Differentiated Papillary Thyroid Carcinomas; Avik Chakraborty, S.V. Kane, M.G.R Rajan*

e. Other Publications and Presentatios:

1. **Fully automated Radiosynthesis of novel [^{18}F] Fluoro ethylated Plumbagin derivative and its feasibility study as tumour imaging agent;** Nandy, Saikat; Chakarborthy, Avik; Pawar, Yogita; Ghosh , Sandipto; Chaudhary, P.R.; Rajan , M.G.R.* ; Journal of Labelled Compounds and Radiopharmaceuticals; Vol 56, Suppl 1, pp S1-S512; P 309 (S396), 9 MAY 2013; DOI: 10.1002/jlcr.3058

2. **Simplified and automated synthesis synthesis of O-(2-[^{18}F] Fluoroethyl)-L-Tyrosine ([^{18}F] FET) using a single pot, two-stage procedure and solid phase extraction purification.** Nandy Saikat, Chakraborty Avik, Pawar Yogita, Moghe S.H. & Rajan M.G.R.* European Journal of Nuclear Medicine and Molecular Imaging; Vol.41, Sup. 2, S151 –S705, Page No. S421 (P222), DOI 10.1007/s 00259- 014- 2901-9, 2014

3. **Fully automated Radiosynthesis of novel 3'-deoxy-3'-[^{18}F]Fluoro Thymidine ([^{18}F]FLT) with anhydro precursor (DMTThy) and Sep-Pak® cartridge purification and analysis of chemical and radiochemical purity.** Nandy, Saikat; Chakarborthy, Avik; Pawar, Yogita; Moghe, S.H.; Rajan, M.G.R.* European Journal of Nuclear Medicine and Molecular Imaging; Vol.41, Sup. 2, S151 –S705, Page No. S346 (PW097), DOI 10.1007/s 00259- 014- 2901-9, 2014

4. **Fully automated radiosynthesis of novel [^{18}F] Fluoro ethylated Juglone derivative snd its feasibility study as tumour imaging agent.** Nandy, Saikat; Chakarborthy, Avik; Pawar, Yogita; Moghe, S.H.; Rajan, M.G.R.***** Asia Oceania J Nucl Med Biol; ISSN: 2322-5726; Volume 2, Supplement 1; P-1-28 (Page No. S36); 2014

5. **^{18}F-Labelled trans-Ferulic Acid and its evaluation as tumour imaging agent**. Nandy, Saikat; Chakarborthy, Avik; Pawar, Yogita ; Moghe, S.H.; Rajan, M.G.R. Asia Oceania J Nucl Med Biol; ISSN: 2322-5726; volume 2, Supplement 1; O-2-7 (Page No: S22); 2014

6. **Synergistic effect of ascorbate in napthoquinone induced cytotoxicity, ROS/RNS generation and inhibition of metastasis *in vitro*;** Sujay Gaikwad, Avik Chakraborty, Savita Kulkarni, M.G.R Rajan*; J Cancer Sci Ther 2014, 6:9 http://dx.doi.org/10.4172/1948-5956.S1.037

7. **Anti-proliferative effect of the Phyto-chemical Plumbagin on human Thyroid Cancer Cell Lines with *BRAF* Mutations;** A. Chakraborty, S. K. K. Sandur, M. Rajan*, A. A. Narkar; Abstract No. A38, 7th NCRI International Cancer Conference organized by National Cancer Research Institute in **Liverpool, UK. (2011) (available online: http://www.ncri.org.uk)**

8. **Apoptotic induction by plumbagin in *BRAF* mutated and wildtype human thyroid cancer cell Lines through mitochondrial complex III mediated ROS/pJNK pathway**; A. Chakraborty, S. K. K. Sandur, M.G.R Rajan*; All India Cell Biology Conference & International Symposium-SARGI", poster no 67, October 17-19, 2012 at Bhabha Atomic Research Centre, Mumbai, India.

9. **Anti-proliferative effect of the NF-kB Inhibitor Plumbagin on human Thyroid Cancer Cell Lines with *BRAF* Mutations**; Chakraborty Avik , Sandur Santosh K[1], Rajan MGR and Narkar Archana A. Presented at 28th Annual Convention of Indian Association for Cancer Research & International symposium on "Emerging challenges and approaches in cancer biology", IISc , Bangalore, 21-24 Feb, 2009

Avik Chakraborty

Date:

Dedicated
To my
Loving parents & parents in-law

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Avik Chakraborty.

Contents

SYNOPSIS

Oncogenic Alterations and their Implication in Thyroid Carcinoma

Introduction:

Thyroid cancer, although relatively rare, is the most common cancer of the endocrine glands. It has a wide range of biological and clinical behavior ranging from very indolent to a highly aggressive form, with an overall mortality of approximately 10 %. It can be classified histologically into follicular epithelial cell derived papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), Follicular variant of papillary thyroid cancer (FVPTC), Follicular thyroid adenoma (FTA), anaplastic thyroid cancer (ATC) and parafollicular C-cell derived medullary thyroid cancer (MTC) which accounts for approximately 80, 15, 10, 3, 2 and 3% of all thyroid malignancies respectively. [1]

It is known that genetic alterations are the driving force for thyroid tumourigenesis and progression and have been associated to specific types of thyroid carcinomas. The classical oncogenic genetic alterations commonly seen in thyroid cancer include RAS mutations, RET/PTC rearrangements, *BRAF* mutations and *PAX8/PPARγ* fusion oncogene. Medullary thyroid carcinoma (MTC**)** is associated with the activating mutations of the RET proto-oncogene. [2, 3]

As mentioned above the wide divergence in clinical behavior of thyroid carcinoma is poorly predicted by current clinico-pathological factors. Hence, different molecular alterations in various thyroid carcinomas, that have been recently identified, are being explored clinically. These molecular alterations have their utility in diagnostic evaluation, staging, prognosis and targeted treatment. Thus, molecular analysis of thyroid cancer promises to increase our understanding of its biologic behavior and is expected to have further impact on its clinical management.

RMC has the largest registries of thyroid cancer patients in India and also in South East Asia. Approximately 500 new cases are being referred every year for staging and ^{131}I therapy as thyroid cancer. However the type and prevalence of various mutations in thyroid cancer patients remain so far unidentified among the Indian patients. It is known that geographic, regional and genetics may contribute to different types of mutations and their frequencies as well as their role in the molecular pathogenesis of thyroid cancer.

Therefore the thesis is aims at:

1. **To study the prevalence of *BRAF* ^{V600E} mutation and its usefulness as a diagnostic and prognostic tool in thyroid carcinoma**
2. **To study the prevalence of *BRAF* mutation in tall cell variant of thyroid carcinoma**
3. **Detection of *RET/PTC* and *PAX8-PPARγ* rearrangements and their correlation with *BRAF* mutation and clinic-pathological parameters in thyroid carcinoma**
4. **Study the prevalence of *RET* (Exon 10,11,13,14,16) gene mutation in Medullary thyroid carcinoma**
5. **study the prevalence of *NRAS, HRAS* and *KRAS* (Exon 1, 2) gene point mutation in Indian subjects**

ORGANISATION OF THE THESIS

The work reported in this thesis is organized into four chapters:

- Chapter 1 deals with general introduction to thyroid carcinoma and the current status.
- Chapter 2 presents the results obtained from study of prevalence of point mutations in proto-oncogenes of MAPK pathway including *BRAF*, *HRAS*, *NRAS* and *KRAS* (Exon 1, 2) and their relevance as diagnostic and prognostic indicators in thyroid carcinoma.

To evaluate *BRAF* mutation and its coexistence with MUC1, HBME and other immune-histochemical markers in poorly differentiated and tall cell variant of thyroid carcinoma real time PCR, sequencing and other molecular analysis were performed and results are described in this chapter.

- Chapter 3 gives the results of the RET/PTC and PAX8-PPARγ rearrangements and their correlation with *BRAF* mutation and clinico-pathological parameters in thyroid carcinoma.
- Chapter 4 describes the prevalence of RET (Exon 10, 11, 13, 14, 16) gene point mutation in Medullary thyroid carcinoma in Indian subjects.

A summary of the work is given at the end of each chapter.

Chapter 1:

Deals with general introduction to thyroid cancer and the published information available in literature related to the present work and its objectives. The characteristics of different thyroid proto-oncogenes and their detection using different molecular typing methods are also described.

Chapter 2:

Prevalence of Point mutations in Follicular Cell Derived Thyroid Carcinomas

2.1 Activating Mutation in *BRAF* Gene in Thyroid Carcinoma

Of all the histological types of thyroid cancer the most prevalent is papillary thyroid cancer (PTC) and accounts for 80-90 % of them. The genetic alterations implicated in PTCs are *RET/PTC* rearrangement, *RAS* mutations and *BRAF* mutations. The aberrant activation of classic signal pathway receptor tyrosine kinase-RAS-RAF-MEK-ERK due to either of these alterations leads to PTC. RAF kinase which is a component of this pathway connects aberrant

activating signals to the cell cycle machinery and plays a key role in transformation. [2, 4] The somatic point mutation of *BRAF* which occurs commonly in a broad range of human cancers, most notably in melanoma, is due to the thymine to adenine transversion at nucleotide position 1799 of *BRAF* gene, resulting in a valine to glutamic acid substitution at residue 600 ($BRAF^{V600E}$). Several studies have been addressed to establish a correlation between $BRAF^{V600E}$ mutation and clinico-pathological features of PTC. However, the results have been controversial. The discrepancies in the results may be due to the different sample sizes including different PTC variants and different geographic areas. [5]

A correct knowledge of the prevalence will help in the unbiased detection of *BRAF* mutation as a diagnostic and prognostic molecular marker for PTC. Hence it is mandatory to build up the knowledge of prevalence of *BRAF* mutation and it's relation with the clinical behavior of PTC in different cohorts all over the world. This will be certainly useful in developing *BRAF* mutation as a molecular marker for the management of PTC in the clinics.

The overall incidence of thyroid cancer in India is 3-4% with 80% of it being PTC. However the type and prevalence of the *BRAF* mutations in PTC patients remains so far unidentified in Indian population. It is known that different geographic areas and genetic background amongst races may contribute to difference in frequency and type of mutations as well as their role in the molecular pathogenesis of thyroid cancer. [5]

The aim of the present study was to establish the prevalence of this genetic alteration and to define association of $BRAF^{V600E}$ mutation with clinico-pathological characteristics of PTC in Indian sub-continental population.

Methodology: Tumour Tissue Specimens: A total of 140 specimens with adequate clinical and pathological information were studied. Tissue specimens included 86 PTC, 14 FTC, 8 FTA, 16 MTC and 16 benign samples. **Cell Lines:** Five thyroid tumour cell lines including

Follicular carcinoma (WRO), papillary carcinoma (NPA), Medullary thyroid carcinoma cell line (TT) and anaplastic carcinoma (ARO and FRO) cell lines were used in this study. **Genomic DNA Extraction:** Nucleic acid extraction from frozen tissues, cell lines and PBMNC was carried out using QIAamp^{R} DNA Mini Kit (Qiagen, Germany) with some modifications. **Single strand conformation polymorphism (SSCP):** Exon 15 amplicons of *BRAF* gene were diluted 1:1 in denaturing solution, boiled for 3 min, rapid chilled, electrophoresed and gels were silver stained. **Mutant allele specific amplification (MASA):** MASA was carried out with two forward primers one complimentary to wild type sequence, another having mutant allele specific nucleotide base substitutions at the 3'end and the common reverse primer. **Sequencing:** *BRAF* exon 15 amplicons were sequenced in a ABI PRISM 377-18 DNA Sequencer (ABI, USA). **Mutector Assay:** Mutector assay was also used to detect *BRAF* mutation at nucleotide position 1799. A detection primer (provided in kit) allowing primer extension in cases of mutant amplicons and preventing primer extension from wild type template.

Statistical analysis: Correlation between *BRAF* mutation and clinico-pathological parameters of the patients was determined by χ^2 - test and nonparametric two-tailed Fisher test and multivariate logistic regression analysis.

Result and Discussion: *BRAF* T1799A (V600E) mutation was found in 46 (53.4%) out of 86 PTC patients (53.4%) studied. The percent incidence of this mutation varied among pathological subtypes of PTC (Table 2). *BRAF* gene mutation was much more common in the conventional PTC (38 out of 62; 61.3%) than in the follicular variants of PTC (2 out of 17; 11.7%). None of the 54 non-PTC thyroid tumours including 8 FTA, 14 FTCs, 16 MTCs and 16 benign samples showed any mutation at this nucleotide position in exon 15. Also, normal thyroid tissue samples taken from the surroundings of the *BRAF* mutation-positive malignant

areas showed no *BRAF* mutations thereby, suggesting that the mutations were somatically acquired.

Thus our findings along with those reported in the literature suggest that morphologic typing in combination with *BRAF* mutation status appears to be better prognostic indicator in PTC. Considering this higher prevalence in Indian cohort, the *BRAF* gene can be a promising target for small molecular inhibitors for better prognosis of radio-iodine refractory thyroid carcinoma patients.

2.2 *BRAF* Mutation in Tall Cell Variant of Thyroid Carcinoma

Tall cell variant (TCV) is one of the aggressive subtypes of PTC which is poorly understood at the molecular level. [6, 7] The recurrence rate is generally high. As this subtype is very rare we are trying to collect at least 30 samples to perform a statistically significant mutation analysis study. One of the patients in our study, a 50 year old lady, showed exceptionally large number of recurrences of this tall cell variant. This is indicative of either a new mutation or partial removal of tumour mass is every surgical intervention.

Methodology: Detailed histopathological and immuno-histochemical analysis of the TCV patients with recurrence is being carried out by an experienced pathologist from TMH. I have performed mutation analysis in *BRAF, RET/PTC, PAX8/PPARγ* genes, transcription level analysis of MUC1, BCL2, BCLXL, Western blot of P53 and immunohistochemistry of Gal3, HBME1, CK19, P27, CyclinD1, ERβ for the primary tumour tissue, metastatic mass and surrounding normal tissue. FDG-PET scan was performed in PET scan facility of RMC.

Result and Discussion: All the tissue samples showed *BRAF* V600E in 100% of the tumour specimen. There is a two fold increase in MUC1 gene at the transcription level. Up-regulation of ERK1/2, NFkβ and down regulation of CyclineD3 was apparent. PET scan revealed ^{18}F-

FDG uptake in metastatic nodules but ^{131}I-scan showed non-iodine concentrating status in case of carcinomas that have recurred.

The present finding in this challenging case supports that multiple local and distant recurrences of the TCV could be related with the genetic mutation in *BRAF*. We believe the findings observed in our patient require to be examined in more number of TCV patients and if proven would open up further research with *BRAF* specific therapies (e.g. BAY 43-9006, AMG 706, Vemurafenib) in this group.

2.3 Prevalence of *H, N* and *K RAS* (Exon 1, 2) Gene Point Mutation

Mutations in the RAS family of proto-oncogenes (comprising *HRAS, NRAS* and *KRAS*) are very common, being found in 20% to 30% of all human tumours. These are recent reports in literature stating that these mutations are seen in FTC and FTA patients in other parts of the world. [8, 9]

Aim: The aim of this work was to detect frequency of sporadic mutation in RAS isoforms (comprising *HRAS, NRAS* and *KRAS*) in thyroid cancer patients included in this study.

Methodology: PCR amplification of exon 1 and 2 of *NRAS, HRAS* and *KRAS* genes were purified and analyzed for SNPs by Competitive Allele Specific PCR method (KASP™, LGC Biosystems, Germany) in 96 tissue samples including PTC, FTC, FTA, FVPTC and PDTC.

10µl aliquots of PCR amplification at 50ng/ml from tissue samples were placed in 96 well PCR Plate. 50 bp upstream and downstream of SNP sites were selected using online Basic Local Alignment search tool (BLAST) in National Center for Biotechnology Information (NCBI). Sealed plates with DNA were sent for analysis using Novel **K**ompetitive **A**llele-**S**pecific **P**CR (KASP) yielding mutation detection with bi-allelic scoring.

Result and Discussion: Sporadic point mutations in *NRAS* [codon 12(GGT>TGT), 13(GGT>TGT), 61(CAA>CTA)], *HRAS* [codon 13(GGT>GAT), 61(CAG>CTG)] and *KRAS*

[codon 12(GGT>GAT), 12(GGT>AGT), 13(GGC>GAC)] were investigated in different histotypes of thyroid carcinomas.

Our investigation reveals that *NRAS* C12GC is prevalent in both PTC (33%) and FTC (28%). Whereas, *NRAS* C13GC is associated only with PTC (33%) making it a promising diagnostic marker for PTC. *HRAS* C13GD and *HRAS* C61QL are prevalently observed in both PDTC (20%, 35%) and FTC (9.5%, 7%) indicating its role in poor prognosis of FTCs and their transformation to PDTC.

Chapter 3:

3.1 PAX8-PPARγ Translocation in Thyroid Carcinoma

PAX8-PPARγ: The peroxisome proliferators-activated receptors (PPARs), including α, β, δ, and γ subtypes, are part of the ubiquitous nuclear hormone receptor super family that has been the focus of considerable research over the last two decades. *PPARγ* plays a role in cell cycle control, inflammation, Atherosclerosis, apoptosis, and carcinogenesis. Research on *PPARγ* as a potential thyroid proto-oncogene was accelerated by the discovery of a chromosomal translocation involving the *PPARγ-1* gene in a subset of follicular carcinomas. These inter chromosomal rearrangement leads to altered *PPARγ* activity and this has subsequently been shown to have a potential role in several types of thyroid cancer. [10]

Following the identification of a frequent translocation in follicular thyroid cancer, involving 3p25 and 2q13, Kroll confirmed not only that the 3p25 breakpoint lies within the *PPARγ* gene, but also that the 2q13 breakpoint lies within the *PAX8* gene. Indeed, the translocation brings together these two genes to form a neogene, which expresses a fusion protein (*PAX8/PPARγ* fusion protein, designated PPFP) [11].

Methodology: Total RNA was isolated from snap-frozen tumour samples using TRIzol reagent (Invitrogen) and HiPura-A Mini Kit (Himedia). cDNA was synthesized from 2µg

RNA with Superscript II reverse transcriptase (Invitrogen). PCR was performed with primers specific for sequences in exon 6 of *PAX8* and exon 1 of *PPARγ* in a mixture containing 2µg cDNA. PCR products were resolved by 5% acrylamide gel electrophoresis, and their sizes were determined by comparison with DNA molecular weight markers (MBI Fermentas).

Result and Discussion: 2 (33.34%) out of 6 tumour samples of FVPTC, 1(16.67%) out of 6 FTA and 2(15.38%) out of 13 FTC samples showed presence of rearrangements of exon 6 of *PAX8* with the first exon of *PPARγ.* However, 2(20%) out of 10 MTC samples analyzed showed presence of *PAX8/PPARγ* rearrangement indicating the presence of small numbers other thyroid carcinoma cells that remain undetected during histopathology tests.

Our investigation revealed a significant association of *PAX8/PPARγ* rearrangement with clinico-pathological parameters including tumour stage ($p<0.05$), metastasis ($p<0.05$) and extra-thyroidal extension ($p=0.009$) making it a significant prognostic molecular marker for our patient population. Whereas, its distribution throughout different histotypes gives it a poor diagnostic value.

3.2 *RET/PTC* Rearrangement in Thyroid Carcinoma

The RET proto-oncogene is located on chromosome 10q11.2 and encodes a cell membrane receptor tyrosine kinase. The ligand independent activation of *RET* results in the stimulation of downstream signal transduction pathways, particularly the Mitogen-Activated Protein Kinase pathway (MAPK), resulting in increased expression of various nuclear transcription factors and thereby increased protein synthesis and tumourigenic transformation of cells.[12]

Methodology: Methodology: *RET/PTC* rearrangements were analyzed by RT-PCR. RNA was extracted using the HiPura-A Mini Kit (HiMedia). Forward primers were designed on the coiled-coil domains of the *RET* fusion partners (H4 and RFG) and reverse primer was for

RET proto-oncogene. 2µg of RNA were reverse transcribed to cDNA and subjected to 40 cycles of PCR. The product was analyzed on a 5% acrylamide gel.

Result and Discussion: Nine (27.27%) out of 33 PTC samples analyzed showed presence of *RET/PTC*1 and *RET/PTC*3 rearrangement. However, there hasn't been any tumour sample that showed *RET/PTC*2 rearrangement. 5 (15.15%) and 9 (27.72%) samples showed coexistence of *BRAF* point mutation and rearrangements *RET/PTEC1* and *RET/PTC*3 respectively. Also 3 (9%) samples showed the coexistence of *BRAF* point mutations with both the isoforms of *RETPTC* rearrangements [13, 14]**.**

Thus our findings along with those reported in the literature suggest that RET gene rearrangement is conserved only in PTC. Its association with *BRAF* mutation makes it a less promising genetic alteration in a multiple marker diagnostic assay.

Chapter 4:

Prevalence of RET gene mutation in Medullary thyroid carcinoma

Alteration of the *RET* proto-oncogene plays a causal role in the familial forms of MTC. In medullary carcinomas, *RET* is activated by point mutation, in contrast to its activation by chromosomal rearrangement in PTC. Germline mutations in the discrete functional regions of *RET* are found in almost all patients with familial forms of medullary carcinoma. In MEN 2A and familial MTC, mutations are typically located in one of five cysteine codons within the cysteine-rich extracellular domain. [15]

In this study efforts have been made to investigate missense mutation in *RET* gene (Exon 10,11,13,14,16) of patients with MTC, both MEN2A and MEN2B and 40 samples have been studied.

Methodology: Genomic DNA was purified using the method previously described. PCR amplification was performed using five sets of primers. PCR amplified products were gel

purified and subjected to dideoxy sequencing using Big Dye Terminator V301 Sequencing Kit.

Result and Discussion: We could find mutations as well as polymorphism at exon 10, 11, 13, 14 and 16 at different frequencies in 25 samples. Genomic DNA extraction and amplification of exons for the remaining samples have been performed.

Novel missense mutations observed in following codon: (Lys595Asn), (Glu605Arg), (Met657Ser), (Lys666Asn), (Ala672Thr), (Glu673Asp), (Phe676Ile), (Ser686Thr) and (phe709Cys), which code for cysteine rich domain, transmembrane domain and tyrosine kinase domain, resulting into upregulation of MAP Kinase pathway.

We observed an overrepresentation of a germ line *RET* sequence variant in exon 10 in 4 (16%), exon13 (L769L) in 12(48%) and exon 14, (S836S), in 1(4%) out of 25 patients among isolated patients affected with MTC, compared with control subjects from the same geographic, ethnic and genetic backgrounds. These preliminary data suggest that the *RET* polymorphism might be playing a crucial role in oncogenesis of MTC observed in our population.

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Abbreviations:

%	-	Percent
^{0}C	-	Degree Celsius
APS	-	Ammonium Persulphate
ATC	-	Anaplastic Thyroid Carcinoma
bp	-	Base pairs
BRAF	-	B Type Raf Kinase
BSA	-	Bovine serum albumin
cDNA	-	Complementary DNA
DEPC	-	Diethyl-pyrocarbonate
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribose Nucleic Acid
dNTP	-	deoxyribonucleotide triphosphate
DTT	-	Dithiothreitol
EDTA	-	Ethylene Diamine Tetra Acetic Acid
EtBr	-	Ethidium Bromide
ETE	-	Extra Thyroidal Extensions
FBS	-	Foetal bovine serum
FNAB	-	Fine Needle Aspiration Biopsy
FTA	-	Follicular Thyroid Adenoma
FTC	-	Follicular Thyroid Carcinoma
FVPTC	-	Follicular Variant of Papillary Thyroid Carcinoma
G	-	Gram
kb	-	Kilobases
kDa	-	Kilodalton
MAPK	-	Mitogen Activated Proliferating Kinase
MUC	-	Mucin 1
MTC	-	Medullary Thyroid Carcinoma
ng	-	Nanogram
OD	-	Optical density
ORF	-	Open reading frame
PAGE	-	Polyacrylamide gel electrophoresis
PAS	-	Para-aminosalicylic acid
PAX8	-	Paired Box 8
PBMC	-	Peripheral blood mononuclear cells
PBS	-	Phosphate buffered saline
PCR	-	Polymerase chain reaction
pg	-	Picogram
PPAR	-	peroxisome proliferators-activated receptors
PTC	-	Papillary Thyroid Carcinoma
RAS		Rat Sarcoma viral oncogenes

HRAS		Harvey Rat Sarcoma viral oncogenes
NARS		Neuroblastoma Rat Sarcoma viral oncogenes
KRAS		Kirsten Rat Sarcoma viral oncogenes
RET/PTC	-	Rearranged during Transfection in Papillary Thyroid Carcinoma
RNA	-	Ribonucleic Acid
RNase	-	Ribonuclease
ROI	-	Reactive oxygen intermediates
ROS	-	Reactive oxygen species
RT	-	Room temperature
RTK	-	Receptor Tyrosine Kinase
RT-PCR	-	Reverse Transcriptase Polymerse Chain Reaction
SDS	-	Sodium dodecyl sulphate
TAE	-	Tris Acetate EDTA
TBE	-	Tris Borate EDTA
TBS	-	Tris buffered saline
TE	-	Tris EDTA buffer
TEMED	-	N,N,N',N'-tetramethylethylene diamine
Tris	-	tris (hydroxymethyl) aminomethane
TSH	-	Thyroid Stimulating Hormone
U	-	unit(s)
UV	-	Ultraviolet
v/v	-	Volume/volume
w/v	-	Weight/ volume
WHO	-	World Health Organization
WT	-	Wild type
β-ME	-	Beta-mercapto-ethanol
µg	-	Microgram
Ml	-	Microliter
µM	-	Micromolar
KASP	-	K(c)ompetitive Allele Specific PCR
TCV	-	Tall Cell Variant of Papillary Thyroid Carcinoma

Chapter 1

General Introduction

1.1 Pathogenesis of Cancer

Cancer is the second most common cause of death after cardiovascular disorders in the world *(1)*. The prevalence of cancer in India is estimated to be around 25 lakhs, with about 7-8 lakh new cases and 5.5 lakh deaths per annum *(2)*. The major types of cancer are carcinoma, sarcoma, melanoma, lymphoma, and leukemia. Carcinoma (in Greek, karkinos, or "crab", and -oma, "growth") is a type of cancer that develops from epithelial cells. Specifically, a carcinoma is a cancer that begins in a tissue that lines the inner or outer surfaces of the body, and that generally arises from cells originating in the endodermal or ectodermal germ layer during embryogenesis.

Cancer is a complex genetic disease that is still being unraveled. Work over the past fifty years confirms that the genetic alterations found associated with human cancers impair the function of pathways critical to controlling cell growth and differentiation. When these mutations aggregate, they allow a malignant cell to acquire a set of biologic attributes leading to autonomous proliferation and metastatic spread. Despite this paradigm, the precise nature and timing of each of the events that conspire to program the malignant cell are yet incompletely understood.

Although familial cancer syndromes are responsible for only a minority of human cancers, the study of these kindred has facilitated our understanding of cancer genetics. In many such syndromes, individuals inherit one defective, predisposing allele in the germ line, and only later in life do they acquire a second loss of function mutation. As first described by Knudson, this “two hit” hypothesis helps explain such inherited cancer syndromes such as retinoblastoma and Wilms’ tumours (3). Although the tumours in these patients express

mutations in specific inherited genes, the finding that these tumors also harbour a myriad of other genetic changes indicates that further alteration by somatic mutation are required for tumour development (4).

However, the majority of human cancers lacks a readily definable predisposing genetic defect and appears to be the result of a several acquired genetic alterations. Work from many laboratories, using both patient-derived material and experimental cancer models have begun to define these malignant genetic mechanisms.

In spontaneously arising human cancers, we still cannot determine the exact number and nature of genetic alterations involved in the process of transformation from a normal cell to a malignant one. Since cancer encompasses more than 100 different types of malignant diseases with great heterogeneity of clinical characteristics, every tumour could hypothetically be completely unique. Thus, cancers, in general, could harbour an undecipherable number of genetic and epigenetic changes leading to their development.

Alternatively, pathogenesis of human cancers may be dependent on a distinct set of genetic and biochemical alterations that apply uniformly to most if not all human tumours. These changes may alter the functions of specific pathways involved in important biological functions and facilitate malignant transformation, endowing cells with specific changes in cell physiology, termed “acquired capabilities,” ensuring their survival and continued success (5). In particular, cancer cells generate their own mitogenic signals, proliferate without limits, resist cell cycle arrest, evade apoptosis, induce angiogenesis, and eventually devise mechanisms for invasion and metastasis.

1.1.1 Genetic Alterations in Cancer

Epidemiologic analyses have shown that four to six rate-limiting events must occur before a tumour becomes clinically apparent (6). The changes that must occur are genetic and/or

epigenetic in nature. Most of these events result from somatic mutations that occur infrequently or are induced by carcinogen exposure, and only in aggregate do they lead to the tumourigenic state.

1.1.2 The carcinoma model

By studying tissue derived from specific histopathologic stages, ranging from normal epithelium to frank carcinoma, genetic alterations specific for each stage has been catalogued. Thereby, a model that analyzes an accumulation of separate genetic mutations could aid in understanding the combination of events that lead to the development of malignancy (7).

Observations in colorectal cancer, suggest that oncogenesis cannot be accomplished by a random accumulation of mutations. The etiology of human cancer follows a stepwise progression of genetic events. This model demonstrates only one of many potential pathways to the neoplastic state. (Figure 1.1).

Similar mapping is yet to be done in most of other human cancer and abundant evidence indicates that specific mutations differ among particular cancers. Understanding the combination of events required in each type of human cancer remains an important goal of future studies.

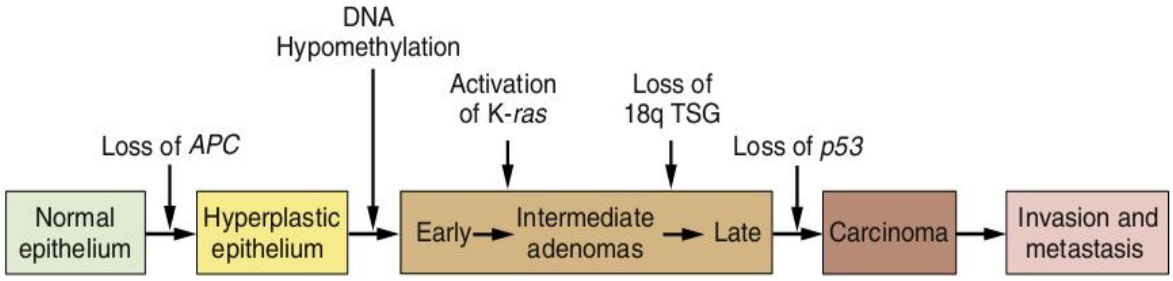


Fig 1.1: By 1990, analyses of the genomes of colonic epithelial cells at various stages of tumour progression revealed that the more progressed the cells were the more mutations they had acquired *(Courtesy: Land H, Parada LF, Weinberg RA. Nature. 1983; 304:596-602)*

1.1.3 Experimental Models

Initial studies of human cancer cells were limited to samples obtained from tumour biopsy specimens. To facilitate further genetic analysis, cells from these tumours were frequently adapted into cell lines that grow in culture (8). However, the high likelihood that additional genetic alterations are acquired over time, through propagation in culture, is a confounding factor in such genetic analysis.

Recently, transcriptional profiling has been helpful in evaluating the simultaneous expression of thousands of genes in particular cancers or cancer cell lines (9). To analyze true functional roles of genes the new approach is to transform normal cells, *in vitro*, by serially introducing multiple oncogenes.

In rodent systems, two introduced oncogenes (*Ras* or *Myc* transgene) converted embryonic rodent cells to a tumorigenic phenotype (10, 11) farther indicated that multiple genetic changes are required for malignant transformation.

While two oncogenes appeared to suffice to transform rodent cells, the transformation of primary human cell lines proved to be more complex. From a set of observations, Sager and her colleagues postulated that the senescence program is a barrier to cancer development in human (12). Recent work, however, has begun to identify combinations of genetic alterations that suffice to confer human cellular transformation.

Studies have revealed this combination of genetic alterations to be sufficient to transform multiple cell types, including cells of mammary, lung, prostate, ovarian, mesothelial, endothelial, and neuroectodermal (13, 14) origin. Thus, it is necessary to understand the roles of these basic genetic elements involved in transformation in regards to the critical pathways that they effect.

1.1.4 Molecular Changes

Although there are many important cellular capabilities, viz., cell cycle arrest checkpoints, apoptosis, guard against crisis, and provide its own mitogenic signals, these may be bypassed or avoided by cells with accumulated mutations (putative cancer cells), and may be sufficient to allow for transformation to the oncogenic phenotype. These well-defined specific molecular alterations involved both in thyroid-specific and general malignant transformation are described below.

1.1.4.1 Tumour suppressor genes

1.1.4.1.1 P53 in Neoplasia

Perhaps one of the most common alterations in human cancers is mutation of the P53 pathway, found altered in most, if not all, human cancers. Loss of wild-type p53 protein expression, in conjunction with gain-of-function from mutant proteins (15), contribute to acquisition of specialized cell properties, such as proliferative and survival advantages.

Wild-type P53 protein may act as a cellular defense mechanism through its effects on cell cycle arrest and apoptosis, both major obstacles to tumour formation. Thus, abrogation of wild-type p53 function may be sufficient in some tumour types to dismantle the apoptotic machinery (16). However, in other tumours, specific components of the apoptotic cascade, such as bcl-2, Akt, or caspases (17), must also be inactivated.

P53 regulates a number of genes involved in the cell cycle including cyclin dependent kinases (CDKs), Hdm2, a negative regulator of P53, which is also positively regulated by p53 protein itself.

While P53 may be directly mutated in over half of all human cancers, in some tumours no P53 mutation is observed, yet other genes in the pathway are altered. For example, Hdm2 can

be overexpressed and antagonize p53 protein function in a variety of cancers, including B-cell lymphomas, melanomas, and breast cancers (18).

1.1.4.1.2 Retinoblastoma (RB) protein

Regulation of passage through the checkpoint of the cell cycle is one of the most important roles of the retinoblastoma protein (19). In its hypo-phosphorylated form, this protein bound to various E2F family proteins and inhibits mitosis by blocking cell cycle entry into S-phase.

RB inactivation is a crucial step in allowing a cell to bypass the checkpoint and continue through the cell cycle. Normally, one of the cyclin D subtypes assembles with and these active holoenzymes phosphorylate RB proteins. Once in a hyperphosphorylated state, RB is unable to bind E2F or HDACs (20) (Figure 1.2).

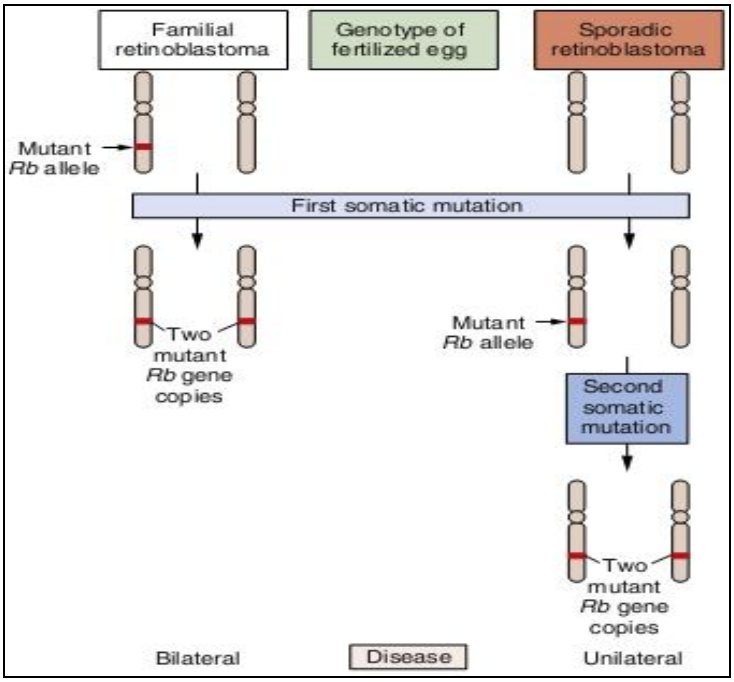


Fig 1.2: Genetics of retinoblastoma development: The development of retinoblastomas requires the successive inactivation of two copies of the chromosomal RB gene (*Courtesy: The Molecular Basis of Cancer,4th ed, John Mendelsohn et. al, 2015, Elsevier*)

Several other tumour suppressor genes including P16, P21 and P27, also contribute to the phosphorylation status of pRB. However, when a strong mitogenic stimulus is present,

increased cyclin D1 helps to phosphorylate and inactivate RB, facilitating cell cycle progression through the G1 restriction checkpoint (21).

Like P53, loss of function mutations of RB is also found in approximately 80% of osteosarcomas and lung cancers, particularly small cell tumours. However, loss is evident in over half of all non-small cell lung cancers. Inactivation of the RB pathway is also observed in a large array of other cancers, including pancreatic, breast, glioblastoma multiforme, and T-cell ALL. In many cervical cancers, human papilloma viruses (HPV) E7 oncoprotein sequesters and tags RB for degradation (22). However, it is reported by Harvey, et al., that mice deficient in both p53 and Rb develop tumours primarily of endocrine origin (23).

1.1.4.2 Mitogenic stimuli and Proto-Oncogenes

Normal and cancer cells differ in their innate ability to proliferate in the absence of mitogenic stimulation. The presence of surrounding growth factors is crucial for the continued proliferation of normal human cells. Cancer cells, in contrast, have reduced their dependence on external stimuli due to the activation of oncogenic mutations that generate constitutively active mitogenic signals (24). For example, alterations or mutation in growth factor receptor (eg. HER2, RET), function as autonomous growth stimuli (25).

1.1.4.2.1 Oncogenic RAS

Since multiple activating mutations and rearranges have been implicated in the pathogenesis of human cancer, various RAS proteins, members of GTP-binding protein super-family have been implicated. These control several crucial signaling pathways that regulate cell proliferation. Their ability to affect downstream intracellular signaling proteins first rely on post-translational farnesylation to localize the RAS protein to the cell membrane. Then the ratio of biologically active RAS-GTP to inactive RAS-GDP depends upon the presence and

activity of various guanine nucleotide exchange factors (GEFs) and their antagonists, GTPase activating proteins (GAPs) (26).

Multiple effector pathways lie immediately downstream of RAS. The RAF family of proteins, which can trigger a cascade of phosphorylating events through the mitogen-activated protein kinase (MAPK) pathway, leads to cell cycle progression. Alterations in the RAS proteins or their downstream effectors can therefore have the potential to lead to constitutively active signals, aiding the oncogenic phenotype.

Activating point mutations of RAS occur in approximately 20% of human tumours, most frequently in pancreatic, thyroid, colorectal, and lung carcinomas, obviating the requirement for the neoplastic cells to encounter external growth stimuli. In general somatic RAS mutations seem to be an early event in human cancer (27).

Three members of the RAS family: the ubiquitously expressed K-RAS, N-RAS, and H-RAS, are commonly found to be activated by mutation in human tumours to the extent of 85%, 15% and <1% respectively. These point mutations all prevent GAP induced GTPase activity, leaving RAS in its active, GTP-bound form (28).

In many cancers that lack RAS mutations, downstream effectors of RAS signaling are frequently altered, leading to acquisition of a similar set of neoplastic attributes. Mutations of the BRAF gene were initially found to be present in around 66% of melanomas and also approximately 12% of colon cancers (29). Amplification of the gene results in PI3K activation in 40% of ovarian tumours, while one of its downstream targets, AKT2, can also be amplified in breast and ovarian carcinomas. Finally, the PTEN tumour suppressor gene, acts as a phosphatase on specific downstream targets of PI3K, such as AKT, inactivating that pathway. PTEN deletions occur in 30–40% of human cancers (30).

Characteristic chromosomal rearrangements of the RET gene result in a constitutively active chimeric receptor, termed (RET/PTC) (Chapter 3.2). Some rare rearrangements of specific TRK tyrosine kinase receptors also cause activation of RAS pathway. These rearrangements and single point mutations in RAS pathway genes contribute to many of the "acquired capabilities" of cancer cells, including unregulated growth, inappropriate survival, invasiveness, and angiogenesis (31).

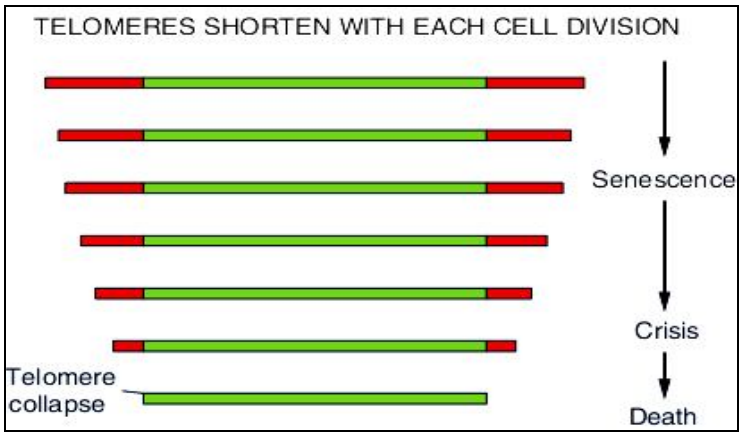


Fig 1.3: Telomere erosion and entrance into crisis *(Courtesy: Shay JW, Zou Y, Hiyama E , Wright WE. Telomerase and cancer. Hum Mol Genet. 2001;10(7):677-685.)*

1.1.5 Telomeres and telomerase

Telomeres are terminal structures at the ends of each eukaryotic chromosome and are composed of guanine rich, DNA repeats, as well as multiple DNA-binding proteins. At the end of each telomere is a single stranded overhang that forms a large secondary loop structure, termed a T-loop. Telomeric DNA is maintained by telomerase, a RNA-dependent DNA polymerase (32, 33) (Figure 1.3).

One of the main functions of telomere is to protect the ends of chromosomes from forming illegitimate fusions, which would lead to genetic instability. In approximately 90% of human tumours, telomere maintenance and replicative immortality may be achieved through activation of telomerase; the remaining tumours may be maintained through "alternative

lengthening of telomeres" (ALT) (34). Interestingly, studies examining malignant transformation in ALT cells lacking P53 and RB function, but expressing oncogenic RAS, confirm that malignant transformation is impossible even with stable telomere lengths unless hTERT is ectopically introduced. Thus, overhang and T-loop maintenance by hTERT may have a role in the mechanism of transformation (35).

1.1.6 Genetic Instability

Although the molecular alterations discussed above, and their regulatory pathways are crucial to the development of a neoplastic cell, one additional hallmark may be necessary to achieve a malignant state. This cardinal feature is genetic instability, which is likely to allow a cell to acquire additional neoplastic attributes more rapidly through the stepwise accumulation of mutations.

When the homeostatic mechanisms that guard the integrity of chromosomes are disrupted, additional genetic alterations may accumulate that lead to further deleterious effects. This instability, however, can be at either a DNA sequence level or at the level of the chromosome, in the form of aneuploidy (36).

Different sets of DNA lesions activate one of the four major DNA repair pathways: mismatch repair, base excision repair, direct repair and homologous/ nonhomologous end joining (37). If these repair mechanisms are not in proper order, a dividing cell could improperly segregate, and depending on the type of lesion, possibly result in aneuploidy. In human cells, the ATR/ATM signaling network, which can together detect a wide variety of DNA lesions through genomic surveillance during DNA replication, has a large role in DNA damage response. ATR disruption is lethal; ATM defects are not and increases cancer risk (38).

Alterations in DNA damage pathways, conferring genetic instability, may be early events in tumourigenesis, as is seen by an increased micronuclei frequency in these tumours. . In a

heterogeneous population of cells, certain cells with the appropriate amount of instability develop a survival and proliferative advantage by selecting out the right set of mutations, typically an accumulation of oncogenes and tumour suppressor genes that are now no longer able to be repaired. This eventually leads to clonal outgrowth and tumour formation (39).

1.1.7 Beyond Cancer Genome

To date, genetic alterations in cancer cells are still the easiest to detect and study experimentally. In the future, the sequencing of an entire human cancer cell genome will certainly yield more important information for the further study of cancer. However, even with this data, many important alterations will be missed, since not all changes occur at the DNA sequence level and are instead occurring at a non-genetic level. For e.g., both epigenetic phenomena and post-translational modifications have critical roles in the regulation of important cellular capabilities that contribute to the neoplastic phenotype.

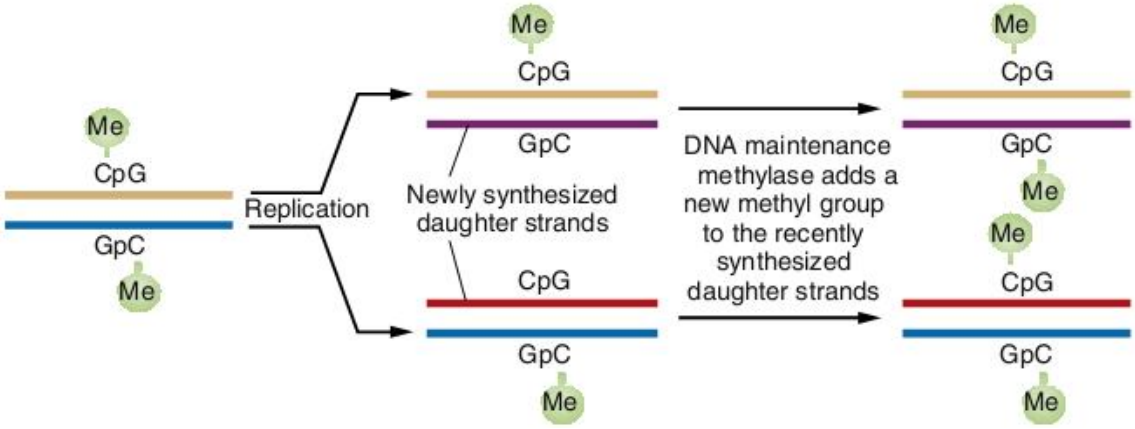


Fig. 1.4: Perpetuation of CpG methylation following DNA replication *(Courtesy: Jones S , Wang T L, Shih I M, et al. Science . 2010;330(6001): 228-231.)*

Epigenetic alterations

Alterations in gene expression that do not involve mutations of DNA sequences are epigenetic events. It has become clear in recent years, that epigenetic changes have an impact

to the development of human cancers through silencing of tumour suppressors and DNA damage elements, chromosomal instability, and even activation of oncogenes (40, 41).

Hyper-methylation mediated silencing of tumour suppressor genes may be important for tumour development since, among other advantages, it tends to lead to a selective cellular growth advantage (41). Methylation of cytosine residues occur at CpG dinucleotides, and 70–80% of these dinucleotides are heavily methylated in human cells (Figure 1.4). Long GC-rich stretches of DNA in the human genome, termed CpG islands, are often uniquely associated with flanking genes and are protected from modification. These normally unmethylated CpG islands may become methylated in cancer cells, resulting in loss of expression of the flanking genes (38). This form of methylation induced silencing affects tumour suppressor genes such as CDH1 and P16, both implicated in cancer development. Epigenetic alterations found in familial and non-hereditary forms of breast and colon cancer offer further supportive evidence for the role of methylation in neoplastic formation (42).

Although the exact mechanism of abnormal epigenetic changes leading to neoplasia is unknown, likely candidates include changes in expression of the key enzymes that regulate DNA methylation, such as the DNA methyl-transferases (DNMTs). Over-expression of DNMT mRNA levels have been found in malignancies of various histological origin, including lung, colon, and ovarian cancer cells. Further evidence is the fact that over-expression of DNMT1 leads to de-novo methylation of CpG islands, and can facilitate cellular transformation (43, 44).

1.1.8 Conclusion:

The development of human cancer is a complex process that involves the multiple gene mutations, translocation, ineffective DNA damage-repair pathways, upregulation of proto-oncogenes and down regulation of tumor suppressor genes, alterations in transcription and

translation factors, indicating the involvement of alterations of multiple cell physiologic functions. Although possibilities for genetic and/or epigenetic alterations are innumerable, common principles have recently been delineated that ensure the success of any cell exhibiting a malignant phenotype. The specific pathways and principles discussed above are known to contribute in an intimate manner to this process, but the foundation is just being set.

Since this thesis is about genetic mutations in thyroid carcinomas, these malignancies offer excellent models for studying cancer pathogenesis in general, as they offer a broad spectrum of tumour subtypes. For instance, papillary tumours tend to be well-differentiated, slow growing tumours that can be treated with radioiodine and thyroxine-suppression, and may have utility in studying early genetic lesions involved in neoplastic formation. On the other hand follicular tumours are more aggressive as they can lose their differentiated nature easily leading to a poor prognosis. At the other end of the differentiation spectrum, are the anaplastic tumours that are very aggressive and difficult to treat resulting in high mortality. Hence, a careful prospective study of these tumours can yield considerable information on the initiation and progression of genetic mutations responsible for the different types of thyroid cancers.

In the following chapters, the specific molecular defects involved in thyroid cancers will be discussed in detail. These defects may be specific for different subsets of thyroid carcinomas, but they typically lead into unifying pathways that phenotypically result in specific “acquired capabilities” for the cells to become malignant.

1.2. Introduction to Thyroid Cancer- current knowledge

Thyroid carcinoma is fairly uncommon, representing only 1 % of all cancers but accounts for more than 90% of all endocrine cancers (45). The thyroid is a complex and fascinating endocrine gland with multiple functions, including regulation of calcium homeostasis and basal metabolism. Thyroid nodules are frequently seen in clinical practice and the majority are benign. Thyroid nodules represent a variety of different thyroid disorders from the non-neoplastic conditions such as goitre or thyroiditis to neoplastic nodules that can be benign or malignant. Sometimes adenomas and goitre are seen in combination with either hyper- or hypothyroidism. Thyroid carcinomas comprise of a diverse group of malignancies ranging from the indolent papillary micro-carcinoma that is hardly any threat to survival, to the rare anaplastic carcinoma that is very aggressive with high morbidity and mortality. The overall incidence of thyroid cancer is reported to have slowly risen, from a rate of 1.3/100,000 women and 0.2/1 00,000 men in 1935 to 5.8/1 00,000 women and 2.5/1 00,000 men in 1991 *(46)*. Based on the survey of the year 2007-2011, the number of new cases of thyroid cancer is 12.9 per 100,000 men and women per year as shown in Figure1.5. In India, thyroid cancer prevalence is higher and is reported to be 3–4% of all cancer patients *(1)*. However enough information regarding association with genetic mutation to the prevalence and prognosis for this disease is not available.

1.2.1 The Thyroid gland

1.2.1.1. Embryology

During embryogenesis, the thyroid follicular cells, originating from epithelial cells, descend from the foramen cecum of the tongue to the anterior neck region and fuse with the neuroendocrine C-cells. Normal thyroid tissue can be found along the thyreoglossal duct forming a third pyramidal lobe. The calcitonin producing cells originate from the fifth pharyngeal pouch that give rise to the ultimobranchial body. This is later incorporated into

the central part of each lobe of the thyroid gland. The thyroid gland accumulates and binds iodine after 11 weeks of gestation *(47)*.

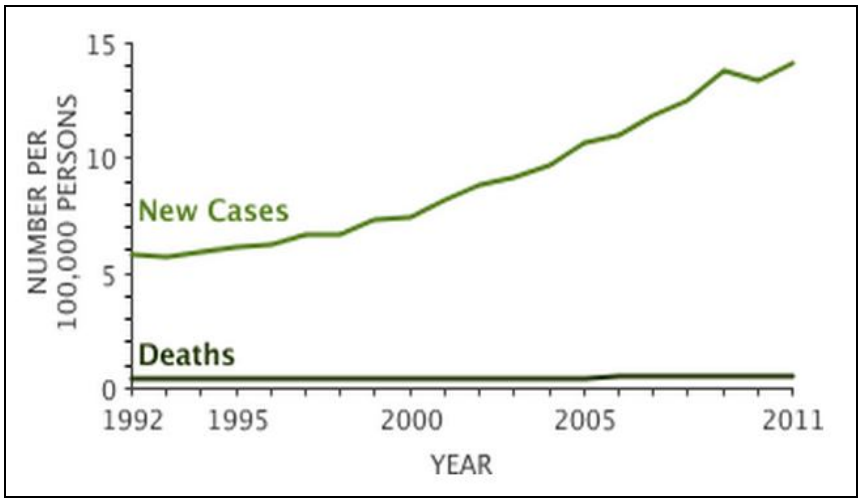


Figure 1.5: Number of New Thyroid Cases and Deaths per 100,000: The number of new cases of thyroid cancer was 12.9 per 100,000 men and women per year. The number of deaths was 0.5 per 100,000 men and women per year. These rates are age-adjusted and based on 2007-2011 cases and deaths.
Source: http://seer.cancer.gov/statfacts/html/thyro.html

1.2.1.2 Anatomy

The human thyroid gland as illustrated in Figure 1.6 is situated in the neck, in front of the trachea and between the cricoid cartilage and the suprasternal notch. Macroscopically, the gland consists of two lobes that are connected by the isthmus. In dog, the thyroid has a similar appearance, while e.g. in birds the two lobes are isolated *(48)*. The blood is supplied mainly by the superior (which is the first branch from the external carotid artery) and inferior thyroidal arteries. Lymphatic drainage is ipsilateral and each lobe can be regarded as a separate entity, although there are some lymphatic anastomoses between the two lobes throughout the isthmus. The recurrent laryngeal nerve, a motor nerve to the intrinsic muscles of the larynx, traverses the lateral borders of the gland. Injury causes paralysis of the vocal cord on the ipsilateral side. The normal weight of the thyroid is 15-30 gram, depending on

body weight and iodine supply. The four parathyroid glands are located in the posterior region of each pole of the thyroid (49).

1.2.1.3. Function

The functional and structural unit of the thyroid gland is the thyroid follicle. This spherical structure is lined with a single layer of epithelial cells and is filled with colloid, which is rich in the thyroid hormonal precursor, thyroglobulin. Between the follicular cells are the parafollicular cells, called C-cells which produce calcitonin, a hormone involved in the calcium homeostasis. The synthesis of the two major thyroid hormones, triiodothyronine (T3) and thyroxine (T4) is a complex physiologic procedure that takes place in the follicular cells. Iodine is an essential element in the thyroid physiology and a critical component in both T3 and T4 molecules. Iodine enters the cells through Sodium Iodide symporter and oxidized with the hydrogen peroxide generated by the enzyme thyroid peroxidase (TPO). It subsequently binds to tyrosine residues of Tg molecule. After conjugation and protelysis of Tg molecule the T3 and T4 hormones are released into the blood at 1:20 ratio. These hormones are transported into different parts of the body in bound (Thyroid binding globulin, Albumin, Transthyretin) or free form (only 0.03 to 0.3%). Only the free form of T3 is biologically active hormone and T4 is converted to T3, either in the thyroid gland or in peripheral tissue cells by deiodinases. Like other steroid hormones, thyroid hormones cross the cell membrane and bind to intracellular receptors (α_1, α_2, $\beta_1$and β_2), and act as transcription factors to modulate DNA transcription related to basal metabolism.

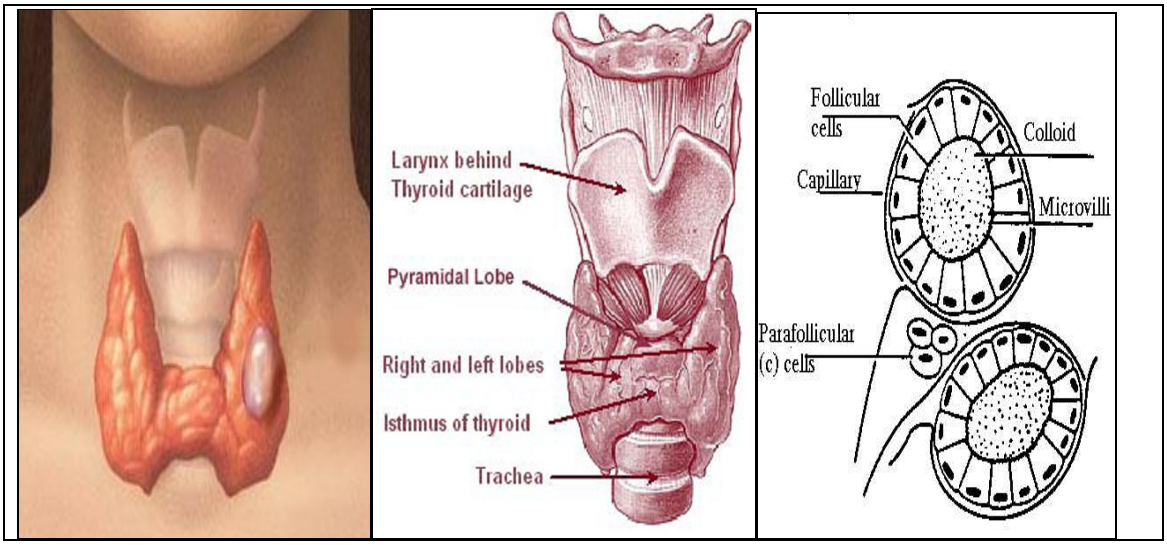


Figure 1.6: The thyroid gland. (Source: http://training.seer.cancer.gov/ & https://twitter.com/ wearecare/status/545658420935020544/photo/1)

T4 and T3 production from thyroid gland is controlled by feedback mechanisms through regulatory hormones from the pituitary and the hypothalamus.. Stimulation to hypothalamus like heat, cold and hunger initiates release of Thyrotropin Releasing Hormone (TRH) which subsequently stimulates the anterior pituitary to release Thyroid Stimulating Hormone (TSH). The production of thyroxine and triiodothyronine is induced by thyroid-stimulating hormone (TSH) by its binding to TSH receptor (TSHR). On the other hand, the TSH production is suppressed by higher levels of T3 and T4 in blood by a negative feedback loop (49).

1.2.1.4. Histology

The normal thyroid gland exhibits a typical histological pattern with spherical sacs (follicles) surrounded by epithelial cells commonly referred to as follicular cells(*8*). The follicles contain colloids with thyroglobulin (Tg) for production of the thyroid hormones, thyroxine (T4) and triiodothyronine (T3). The production of thyroid hormones is regulated by thyroid-stimulating hormone (TSH) from the pituitary gland, which in turn is modulated by various influences such as T4 and iodine levels, thyrotropin-releasing hormone (TRH) from the hypothalamus, and different other hormones. Differentiated thyroid cancer usually secrets Tg into the blood which is a valuable tumour marker for post-operative follow-up.

The parafollicular cells, called C-cells because they produce calcitonin. The functions of calcitonin are related to the metabolism of calcium and phosphorous. High levels of calcitonin are common in patients with medullary thyroid carcinoma (MTC), as such it is used as a marker in MTC diagnostics (49,50).

1.2.1.5. Thyroid tumours

Nodules in the thyroid gland are common, the majority of which are benign (51). In regions with sufficient iodine intake, 4-7% of the general population has clinically detectable thyroid nodules. Thyroid nodules may reflect a variety of non-neoplastic conditions such as thyroiditis or goiter, as well as benign or malignant tumours. Figure 1.7 illustrates the major types of thyroid tumours, recognized in the WHO classification (52). The most common type of tumour is follicular thyroid adenoma (FTA), originating from the follicular epithelium of thyroid. In contrast to nonneoplastic thyroid nodule, FTA is an encapsulated solitary tumour and usually occurs in multiple numbers. FTA is classified as benign, and the diagnosis requires the absence of vascular and capsular invasion (53).

Other subtypes of follicular tumours are; atypical follicular thyroid adenoma (AFTA) and Hürthle cell adenoma (HTA) (52). The diagnosis AFTA refers to adenomas with high cellularity, irregular growth pattern against the capsule and increased mitotic activity. HTA, or oxyphilic cell adenoma, is composed of more than 75% eosinophilic cells with a high content of mitochondria. Malignant thyroid tumours are grossly categorized as well differentiated (WDTC), poorly differentiated (PDTC) or undifferentiated (Anaplastic thyroid cancer; (ATC)) cancer. In general patients with WDTC have superior prognosis as compared to those with PDTC or ATC (54).

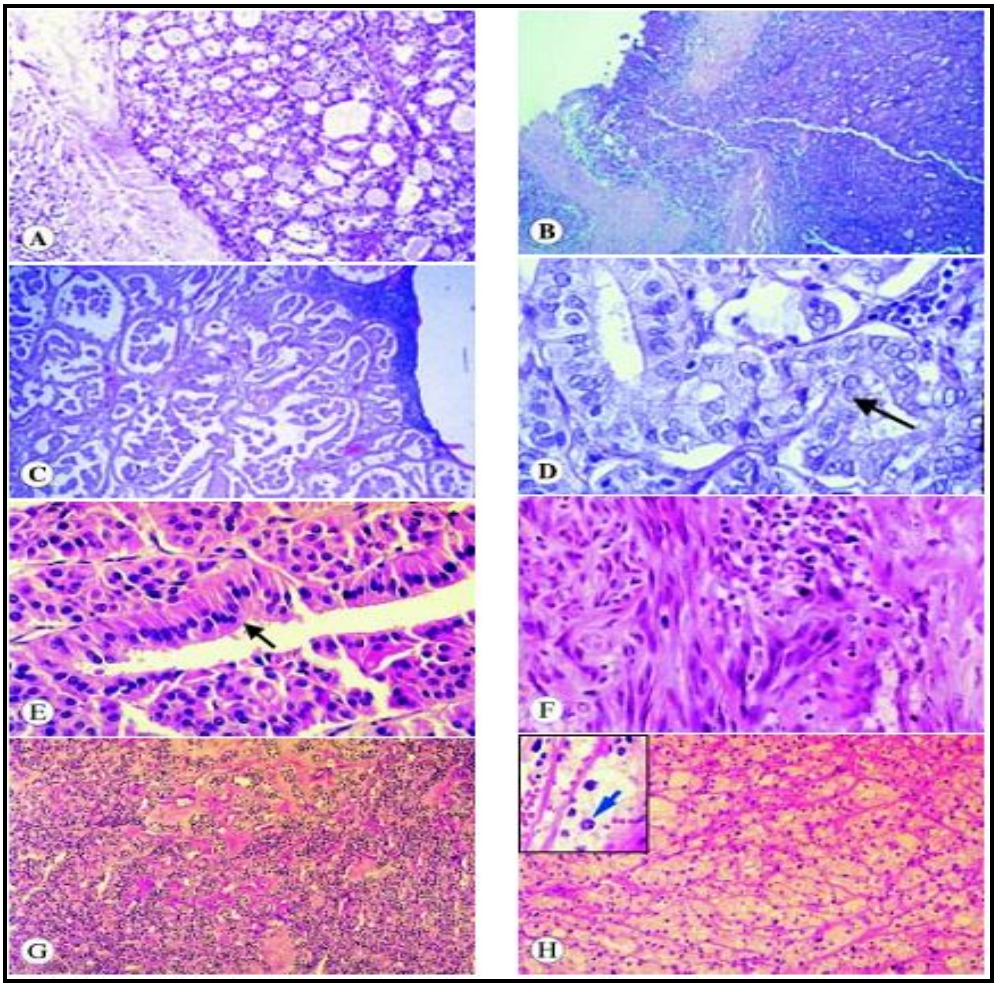


Figure 1.7: Histology of Thyroid Tumours. A. Follicular adenoma: note the sharp separation of a follicular tumour from the surrounding tissue by a uniform fibrous capsule. **B.** Follicular carcinoma with capsular penetration. **C.** Papillary carcinoma metastatic to a lymph node: typical appearance of papillary carcinoma with complex and branching papillae. **D.** Higher magnification showing optical clear, overlapping and grooved (arrow) nuclei. **E.** Tall cell variant papillary carcinoma, lined by tall cells (arrow). **F.** Undifferentiated carcinoma with elongated tumour cells. **G.** Medullary carcinoma. **H.** Papillary carcinoma with clear cell changes: typical intranuclear inclusion (inset).
Source: http://www.hormones.gr/89/article/article.html

Papillary thyroid cancer (PTC)

Papillary thyroid cancer (PTC) is the most common type of thyroid cancer, constituting 80 to 90% of all thyroid cancers *(55)*. PTCs are tumours of follicular origin that exhibit papillary and/or follicular growth pattern and characteristic nuclear inclusions and overlapping tumour nucleus *(52)*. The crucial diagnostic feature for a PTC is the typical nuclear changes (also

referred to as “ground-glass nuclei”). PTC frequently invades lymphatic vessels and lymph nodes.

Follicular thyroid cancer (FTC)

The second most common type of thyroid cancer is follicular thyroid cancer (FTC) comprises about 10% of all cases *(55)*. FTC presents with capsular and/or vascular invasion. Based on the degree of invasion FTCs are further classified as minimally-invasive or widely-invasive *(52)*. Hürthle cell thyroid carcinoma, also known as oxyphillic or oncocytic carcinoma consists of more than 75% oxyphilic cells, is a variant of FTC. FTC metastases are found in the lungs and bones following hematogenous spread.

Medullary thyroid cancer (MTC)

Medullary thyroid cancer (MTC) originates from the calcitonin-producing parafollicular cells and accounts for about 3% of all thyroid cancers *(55)*. Microscopically these tumours are composed of spindle-shaped, round or polygonal cells separated by fibrous stroma that may contain amyloid . MTC may occur in sporadic as well as hereditary forms. An important part of the diagnostic work is the demonstration of immunoreactivity for calcitonin. Metastases to regional lymph nodes are common. Distant metastasis occurs in 20% of the patients and then to the liver, lung and skeleton *(52)*.

Poorly differentiated thyroid cancer (PDTC)

PDTC is a rare type of thyroid cancer, accounting for up to 7% of all thyroid cancer *(55)*. However the real frequency is somewhat uncertain, which is due to related inconsistent definition of this entity. According to the WHO classification *(52)*, PDTC is characterized by increased mitotic activity, tumour necrosis, capsular and vascular invasion and at least focal positive staining for Tg. However, PDTC lacks several morphologic characteristics that are typical for WDTC, suggesting that it may represent an intermediate entity in the progression from WDTC to ATC *(53, 54)*.

Anaplastic thyroid cancer (ATC)

ATC, the most aggressive and lethal form of thyroid cancer, accounts for only 1-2% of all thyroid tumours *(56)*. The prognosis is extremely poor with a median survival of less than one year after diagnosis *(55)*. Previous studies have reported that ATC can occur concurrently with a variety of thyroid disorders, including WDTC *(57)*. ATCs are rapid growing unencapsulated tumours that infiltrate the surrounding soft tissues of the neck and into the respiratory tract.

Microscopically, three types of histologic variants are observed including spindle, giant cell and squamoid cell pattern *(54)*. ATCs are further characterized by frequent mitoses, large areas of necrosis, hemorrhagic, and vascular invasion *(52)*. Staining for Tg is typically negative. In addition, ATC cells do not have thyrotropin receptors or Sodium Iodide symporter and do not secrete Tg. Immuno histochemistry (IHC) staining for calcitonin, chromogranin A, carcinoembryonic antigen and Tg are typically negative. At presentation it is commonly accompanied with regional lymph node metastases, local invasion of surrounding tissues (*i.e.* fat, trachea, muscle, esophagus and larynx), as well as distant metastases in the lung, skeleton and brain. If left untreated most patients will die of ultimate airway obstruction caused by the rapid and extensive tumour growth.

1.3. Thyroid Epidemiology, Diagnosis & Treatments

1.3.1. Epidemiology and risk factors

The incidence of thyroid carcinoma is influenced by several factors such as gender, race and geographical location. Worldwide statistics show women to be more frequently affected than men. Between 1.2 to 2.6 men and 2.0 to 3.8 women per 100,000 individuals are diagnosed with thyroid cancer each year *(58)*. The geographical variation is exemplified by the two-fold higher incidence in Iceland and Hawaii as compared to other North European countries,

Canada, U.S.A and Israel *(59)*. Furthermore, thyroid cancer is also more common among certain ethnic groups, *e.g.* Chinese men, Filipino women in Hawaii *(60)* and Caucasian men and women *(61)*.

Epidemiological studies have suggested that the risk of developing thyroid cancer is influenced by a variety of internal and external factors. Additional determining parameters are iodine deficiency, diet, and exposure to ionizing radiation *(62)*, sex hormones *(63)*, age and gender *(59)*. Individuals with dietary iodine deficiency frequently develop goiter as a result of compensatory thyroid proliferation. Interestingly FTC is more prevalent in areas of iodine deficiency, and ATC often coincides with longstanding goiter *(64)*. Radiation is another well-established risk factor for development of, in particular PTC, which have become evident in individuals exposed to high levels of radiation (*e.g.* Hiroshima, Nagasaki and Chernobyl) *(62)*.

1.3.2. Diagnosis and treatments

When a patient is diagnosed with a thyroid nodule, the first diagnostic procedure performed is the fine needle aspiration biopsy (FNAB) to determine if the nodule is malignant. Most often the cytological analysis by FNAB shows the nodule(s) to be benign: such as colloid goiter, or thyroiditis since the frequency of nodules being malignant is quite low. Some types of thyroid malignancy like PTC and ATC can be readily diagnosed by FNAB, however, in the case of follicular tumours FNAB cannot be used to distinguish between benign and malignant forms.

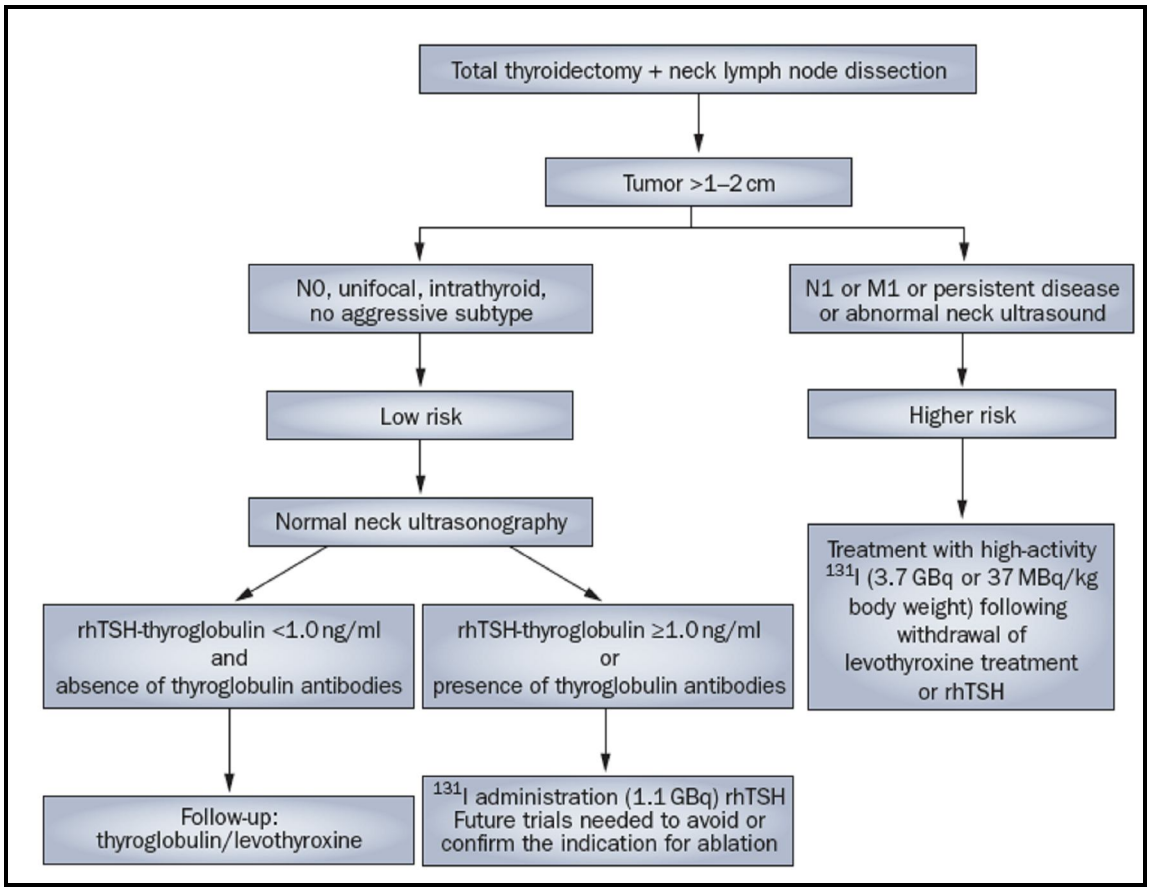


Figure 1.8: Algorithm for the administration of radioiodine in patients with thyroid cancer: modifications of the European consensus. A general consensus exists that tumours <1 cm in size, which are unifocal, intrathyroid and stage N0 do not require radioiodine treatment. Abbreviations: Ab, antibodies; LT_4, levothyroxine; rhTSH, recombinant human TSH; Tg, thyroglobulin.
Source: Nascimento, C. *et al. Endocr. Relat. Cancer 18, R29–R40 (2011)*

This is related to the diagnostic criteria of FTC requiring the demonstration of capsular and/or vascular invasion. Therefore, all follicular thyroid tumours will be treated by surgery, most commonly by removal of the involved lobe (*i.e.* lobectomy) or total thyroidectomy. If the histopathological investigation of the surgically removed tumour shows FTC, a second operation is often performed (*i.e.* total thyroidectomy). There used to be a debate, several years ago, as to whether lobectomy or total thyroidectomy should be recommended for patients with thyroid cancer. The recommendation of total thyroidectomy is supported by reports showing lower recurrence *(65)*, and mortality *(66)* and improved survival *(67)* as

compared to lobectomy. Total thyroidectomy also offers the possibility of effective I^{131} treatment and subsequent recurrences can be identified by measurements of Tg or calcitonin as tumour markers. However total thyroidectomy has the drawback of requiring lifelong substitution with thyroid hormone. On the other hand, lobectomy has the advantage of decreased risk for surgical complications such as hypoparathyroidism and damage to local nerves. It is expected that the increased molecular and genetic knowledge of these tumours will lead to the development of diagnostic tools for pre-operative distinction of these entities. Treatment of ATC remains a challenge in clinical practice. Since the tumours are large, locally infiltrative causing air-way obstructions radiotherapy and chemotherapy are commonly given to decrease the tumour mass prior to surgery. This procedure has both positive effects on the outcome of surgery as well as reduces the local symptoms in the neck *(56)*. The algorithm for the administration of radioiodine in the patients with thyroid cancer by the Society for Endocrinology is provided in Figure 1.8.

1.4. Molecular Genetics of Thyroid Tumours

1.4.1. Two hypotheses of thyroid carcinogenesis: multi-step carcinogenesis and fetal cell carcinogenesis

Since the 1980s, cancer cells, including those of the thyroid, have been considered to be derived from normal cells, such as thyrocytes, via multiple incidents of damage to their genome, especially to the oncogenes or tumour suppressor genes, which accelerate uncontrolled proliferation and metastasis. According to multi-step carcinogenesis hypothesis, follicular carcinomas are generated from follicular adenomas, while papillary carcinomas are derived from some unknown precursor cells generated by normal thyrocytes. Anaplastic carcinomas are generated by both follicular and papillary carcinomas by genomic changes, such as mutations in the *TP53*, as described in figure 1.9. The existence of common genomic changes between differentiated carcinomas and anaplastic carcinomas offer direct proof of

the multi-step carcinogenesis hypothesis. *RAS* and *BRAF* mutations are frequently observed in thyroid carcinomas. On the other hand, micropapillary carcinomas, which are often observed in autopsies, show a distinct morphological difference from thyrocytes *(68)*. It is hard to believe that these carcinoma cells obtain their cancerous characteristics via multi-step carcinogenesis, since thyrocytes rarely proliferate and papillary carcinoma cells are very slow to grow. In 2000, another hypothesis of thyroid carcinogenesis was proposed, the "fetal cell carcinogenesis", in which cancer cells are derived from the remnants of fetal thyroid cells instead of thyrocytes *(69)*. A considerable number of researchers have come to believe that cancer cells are derived from immature progenitor or stem cells, but not from well-differentiated cells *(70-73)*.

Development of thyroid tumours is associated with a variety of molecular and genetic mechanisms. It is related to oncogene activation, fusion oncogenes, tumour suppressor inactivation, genomic imbalances, expression profiles of genes, proteins, microRNAs, epigenetic modifications, and possibly mitochondrial DNA alterations. Several of these modifications are associated with certain types of thyroid tumours or related to familial and sporadic forms of disease as discussed below. The expanding knowledge of molecular genetics in thyroid cancer, continuously adds to our understanding of thyroid tumour etiology.

1.4.2. Oncogenes

Dominant stimulatory genes that cause cancer are termed oncogenes. Many of the components of mitogenic signaling pathways are encoded by genes that were originally identified as cancer-promoting genes or oncogenes because mutations in them contribute to the development of cancer. Activating oncogene mutations are frequently encountered in thyroid tumours and may be observed in germ-line and more commonly on the somatic level.

Well-known examples of recurrently involved oncogenes are *BRAF*, *RET*, the three RAS genes, β-catenin and PIK3CA which are described separately as follows.

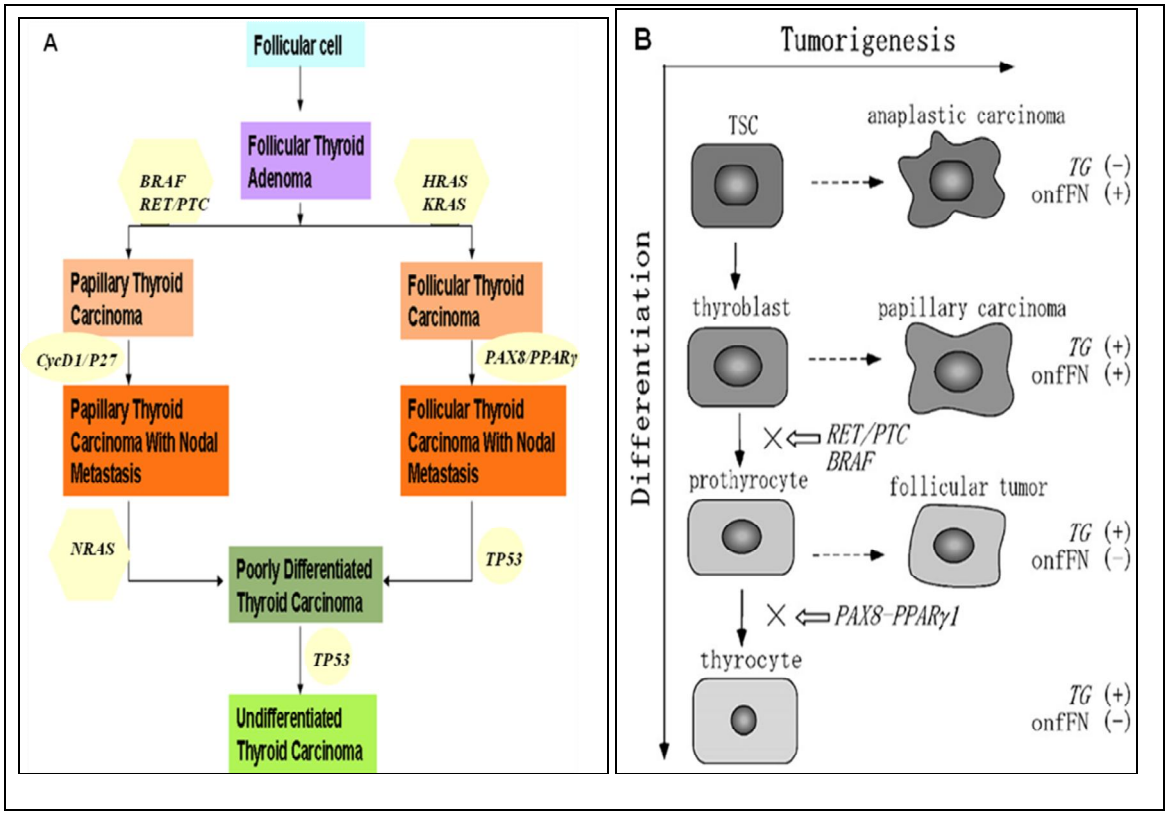


Figure 1.9: Multi-step and Fetal cell carcinogenesis. **A.** According to multistage carcinogenesis theory, different histological subtypes of thyroid carcinoma are generated from a common thyroid follicular cell (thyrocyte) and dedifferentiate depending of acquired mutation. **B.** Feta cell theory proposes that different histology of thyroid cancer originate from thyroid cells present at different stages of differentiation by accumulation of mutation at those specific stages. (Source: Takano, T. (2007) Fetal cell carcinogenesis of the thyroid)

RET

The *RET* (*rearranged during transfection*) proto-oncogenes *(74)* located in chromosomal region 10q11.2 encodes for a tyrosine kinase receptor that is expressed in the cell membrane of different tissues including the thyroid, adrenal medulla, parathyroid, and enteric ganglion *(75)*. The *RET* receptor consists of an extracellular, one transmembrane, and two intracellular domains *(76)*. This particular gene has been strongly associated with the development of thyroid cancer *(75)*. Activation is achieved by binding of the complex formed by the glial cell

line-derived neurotrophic factor (GDNF) ligand and the GDNF family receptors-α (GDNFα). Binding of the ligand induces dimerization of the receptor and autophosphorylation in the intracellular domain, where after downstream signaling cascades can be activated *(76, 77)*. The detailed information on *RET* is described in section 3.2 of the current thesis. In MTC *RET* mutations are frequently encountered in exons 10, 11, 13, 14, 15 and 16 *(75)*. These mutations typically involve a single base-pair substitution leading to a shift in amino acid often involving a cysteine residue. MEN 2A or FMTC patients harbour constitutional mutations that are commonly affecting the cysteine-rich part of the extracellular domain or the intracellular tyrosine kinase domain *(78, 79)*. A subset of apparently sporadic MTC cases also carry germ-line mutations of *RET (78, 79)*. MEN 2B is most commonly caused by a mutation in exon 16 that affects codon 918 and the intracellular substrate recognition pocket in the catalytic core *(75)*. Sporadic MTC frequently exhibit the same codon 918 mutation on the somatic level although other mutations may also occur.

BRAF

BRAF mutations are presently known as the most common genetic abnormalities in PTC. The *BRAF* gene is located in chromosome 7, and its encoded protein is a member of the RAF family of serine-threonine kinases *(80)*. *BRAF* normally functions as an effector of the MAPK (mitogen-activated protein kinases) pathway. Binding of Ras leads to activation and recruitment of *BRAF* to the cell membrane for subsequent phosphorylation and activation of members of the MAPK signaling cascade with effects on cellular differentiation, proliferation and survival *(80)*. *In vitro* studies with benign thyroid cells have documented an important function for *BRAF in vitro* studies *(81)*. *BRAF* is frequently mutated in a variety of different human cancers such as melanoma, ovarian cancer and colorectal cancer *(80)*. One of the hitherto reported mutations constitutes approximately 90% of all mutations. This “hotspot” mutation involves a single nucleotide transition in exon 15 (1799T-A), predicted to give an

amino acid substitution of a valine by a glutamic acid at the position 600 (V600E) *(82)*. The missense mutation has been reported in approximately 45% of PTC *(83)*, thus making it the most common genetic abnormality of this entity. Although V600E is by far the most common type of mutation, activating *BRAF* mutations have also been reported at codons 599 and 601*(84)*. Furthermore, V600E has also been found in approximately 10% of PDTC and a third of ATC *(55)*. In ATC, *BRAF* mutations have been reported to be more prevalent in cases presenting with a PTC component as compared to regular ATC. Based on these results, it has been hypothesized that PTCs with a *BRAF* mutation would be more aggressive and could possibly progress to ATC. However, the possible relationship between V600E and aggressive tumour phenotypes is controversial *(85)*. The more detailed studies are described in section 2.1 of the current thesis.

RAS

Point mutations in any of the three *RAS* genes *H-RAS*, *K-RAS*, and *N-RAS* are common findings in human cancers and present estimates suggest that approximately 30% of all human tumours harbour mutations in one of these genes *(86)*. *RAS* mutations are also commonly observed in follicular thyroid tumours including both FTA and FTC, while in PTC it is less frequent with the exception of PTC of follicular variant. *RAS* mutations have also been reported in PDTC (18-27%) and ATC (20-60%). These findings could possibly suggest a progression of *RAS* mutated follicular tumours to ATC, but may also be coincidental following the overall high frequency of *RAS* mutations in human cancer. *RAS* mutations are typically missense alterations that affect two different locations in the gene including: exon 1 and codons 12 or 13 of the GTP binding part; or exon 2 and codon 61 of the GTP-binding domain *(86)*. The normal function or Ras molecules are to convey signals from membrane-bound tyrosine kinase receptors to the MAPK cascade to activate transcription of target genes. Following activation of *RAS* by mutation, downstream targets will be continuously

stimulated with effects *e.g.* apoptosis and proliferation, and in addition contribute to genomic instability, mutations and malignant transformation *(86)*.

β-*catenin (CTNNB1)*

β-catenin encoded by the *CTNNB1* gene is a cytoplasmic protein with several functions. These involve regulation of cell-cell adhesion by binding to cadherins, mediation of the wingless (Wnt) signaling pathway, and transcriptional up-regulation of different oncogenes such as *Cyclin D1* (*CCND1*), *C-MYC* and *C-JUN (87)*. Mutations of *CTNNB1* have been reported in the most aggressive forms of thyroid cancer including 0-25% of PDTC and up to 66% of ATC. *CTNNB1* mutations, especially those in exon 3, stabilize β- catenin so that its normal degradation by the adenomatous polyposis coli (APC) complex is avoided *(87)*. This will in turn lead to over-expression of β-catenin and constitutive activation of target gene expression.

PIK3CA

The phosphatidylinositol-3-kinase (PI3K)/Akt signaling involved in regulation of cell growth, proliferation, and survival, has an established role in human tumour development *(88)*. Oncogenic mutations or gene amplifications have been demonstrated in the catalytic subunit of the PIK3CA isoform *(89)*. In thyroid cancer, *PIK3CA* mutations and copy gain have been reported *(90-93)*, with a preferential involvement of *PIK3CA* mutations in FTC and ATC, and of copy number gains in ATC. In a recent study, significant correlation of *PIK3CA* copy gain with increased PIK3CA protein expression was demonstrated *(93)*. In addition, activation of the PI3K/Akt pathway has also been implicated in thyroid cancers *(94-96)*. Notably PIK3CA is activated by Ras, whilst the *PTEN* tumour suppressor has a phosphatase activity antagonizing the signaling of the PI3K/Akt pathway *(88)*. These observations collectively suggest that PI3K/Akt/MAPK pathway is strongly associated with thyroid tumourigenesis.

1.4.3. Tumour Suppressors

p53

p53 is the tumour suppressor gene most frequently mutated in human cancer and it plays a central role in cell cycle regulation, DNA repair and apoptosis. In thyroid cancer, mutations of *p53* are generally restricted to the entities of PDTC (17-38%) and ATC (67-88%) *(55)*. Furthermore in a study of tumours with both WDTC and ATC, *p53* mutations were only detected in the ATC component *(97)*. In animal models, transgenic mice with *RET/PTC* rearrangements that developed PTC were crossed with *p53*-/- mice, giving rise to progeny with rapid development of PDTC and ATC *(97)*. Taken together, these observations indicate that *p53* inactivation is an important step for the dedifferentiation and progression of thyroid cancers.

p27

p27 or kinase inhibitor protein 1 (KIP1) encodes a nuclear protein member of the CDK inhibitors (CDKIs). These molecules negatively regulate cyclin activity and thus control the transition from the G1 phase to the S phase in the cell cycle as reviewed by Park and Lee *(98)*. Several studies have compared p27 levels in thyroid tumours with those of normal thyroid tissues. They reported recurrent decrease in levels of p27, with lowest intensity detected in PDTC *(99)*. Moreover, under-expression of p27 in PTC has been reported as an independent predictor of lymph node metastasis *(100)*.

PTEN

PTEN is a dual-function lipid phosphatase that exerts its tumour suppressor effects by acting on the PI3K and the Akt/PKB pathways *(88, 101)*. Somatic mutations in *PTEN* are rare in primary epithelial thyroid tumours. However hemizygous deletion occurs in 10-20% of FTAs and thyroid carcinomas *(102)*. Moreover, progressive loss or reduction of PTEN

protein expression is observed in thyroid cancers *(103, 104)*. Recently aberrant promoter methylation for *PTEN* was also shown in thyroid cancers *(104)*.

RASSF1A

The *Ras Association Domain family I* (*RASSF1A*) gene is located in chromosomal region 3p21.3 that is frequently lost in many human cancers including thyroid. It is a Ras effector possessing tumour suppressor properties through its involvement in apoptotic signaling, stabilization of microtubule and mitotic progression *(105)*. *RASSF1A* promoter hyper-methylation and LOH have been demonstrated to be the main mechanisms to inactivate this gene, whilst mutation is common in many human cancers. *RASSF1A* methylation but not LOH has been reported in all types of thyroid cancer, mostly in FTC *(106-108)*.

Chromosomal rearrangements

Chromosomal rearrangements leading to oncogenic activation contributing to cancer development have been frequently described in hematological malignancies and sarcomas. In solid tumours of epithelial origin such rearrangements are less well characterized and have previously been regarded as of minor importance. However, thyroid tumours serve as a good example of the importance of chromosomal rearrangements and associated cancer genes in solid tumours. Approximately twenty different such rearrangements are presently known that are described below.

RET/PTC rearrangements

Under normal conditions *RET* is highly expressed in C-cells but not in follicular cells of the thyroid. In thyroid tumours *RET* is frequently activated by mutational events that contribute to tumour development. In PTC *RET* is recurrently activated by chromosomal rearrangement such as translocation or inversion. Following the rearrangement *RET* and the partner gene are juxtaposed leading to fusion of the 3' part of *RET* gene with the 5' part of one of several unrelated genes, known as *RET/PTC* rearrangements. To date, at least 11 different *RET*/PTC

rearrangements have been reported in varying frequencies of PTC cases *(77)*. Overall *RET/PTC* rearrangements have been reported in varying frequencies of PTC cases with more frequently reported in pediatric patients (up to 80%) as compared to adult patients (2-34%) *(97)*. *RET/PTC1* and *RET/PTC3* are the most commonly observed variants among the pediatric patients, which has been proposed to reflect an association with radiation exposure *(97)*. *RET/PTC* rearrangements are regarded as early events in development of PTC but unrelated to progression from PTC to PDTC or ATC *(109)*. Santoro and colleagues found less than 10% of *RET/PTC* positive PDTCs suggesting that *RET/PTC* carrying PTCs have a lower risk of invasion and metastasis *(110)*.

NTRK1 rearrangements

The known chromosomal rearrangements in PTC involve either *RET* or *NTRK1*, which are fused with a variety of different partner genes. The *NTRK1* gene (neurotrophic tyrosine kinase receptor 1) is located on chromosome 1q21-22 and its encoded product is the nerve growth factor receptor. Three different variants of rearrangements involving *NTRK1* have been reported in PTC *(110)* especially in those associated with previous radiation. *NTRK1* rearrangements have been reported in frequencies from 3 to 12% making it less common than *RET/PTC (112, 113)*.

BRAF rearrangement

BRAF presents a similar situation as *RET* in that both activating mutations and chromosomal rearrangements may render an oncogenic function. A novel fusion gene between *AKAP9* (*A-kinase anchor protein 9*) and *BRAF* was recently reported in a small subset of PTCs *(114)*. The underlying chromosomal mechanism is an intrachromosomal rearrangement of chromosome 7 involving a paracentric inversion of the long arm. This resulted in the formation of a fusion gene with in-frame fusion of the N-terminus of *AKAP9* with the C-terminal of *BRAF*. In support of its oncogenic function *AKAP9-BRAF* was shown to have

constitutive kinase activity, to stimulate MAPK pathways and to promote transformation of NIH3T3 cells *(114)*.

PPARγ rearrangements

Chromosomal translocations giving rise to fusion oncogenes are recognized in both PTCs and follicular thyroid tumours. The first fusion gene identified in FTC results from a chromosomal translocation t(2;3)(q13;p25). This leads to fusions of the *PAX8* (*paired domain 8*) gene in 2q13 with the *peroxisome proliferator-activated receptor* γ (*PPARγ*) gene in 3p25 as originally reported by Kroll *et al (115)*. *PAX8-PPARγ* has been identified in FTC in significant but varying frequencies, and in addition it has been reported in a subset of FTAs *(97)*. Chromosomal region 3p25 has also been found to be involved in other translocation events in follicular thyroid tumours, suggesting that it could be a hot spot breakpoint region *(116, 117)*. In two such cases the 3p25 breakpoint has been shown to involve the *PPARγ* gene locus, including one primary FTC *(117)* and one FTA cell line *(116)*. Interestingly reduced expression of the RAS effector *NORE1A* (*RASSF5A*) in FTCs has been associated with the presence of *PAX8-PPARγ (117),* suggesting a link to the *RAS* signaling pathway.

THADA rearrangement

Cytogenetic investigations of FTA have revealed frequent rearrangements involving chromosomal regions 19q13 and 2p21, of which the latter has been associated with the *THADA* (thyroid adenoma-associated) gene *(118)*. The identification of *THADA* was achieved by detailed characterization of two FTA cell lines carrying the translocations t(2;20;3)(p21;q11.2;p25) and t(2;7)(p21;p15), respectively *(118)*. In these FTA cells, *THADA* was found to be fused with sequences from chromosomes 3 and 7. The exact identification of the possible partner genes remains to be clarified, although the 3p25 breakpoint was assigned

to the location of *PPARγ (116)*. The function of the THADA protein is largely unknown but has been suggested to be related to the death receptor pathway *(118)*.

1.4.4. Mitochondrial DNA (mtDNA)

The mtDNA is relatively small and encode for some of the components that are involved in mitochondrial energy-production. The possible involvement of mtDNA in thyroid cancer is by far less well studied than the nuclear DNA. Nevertheless, studies of the mtDNA have revealed deletions and/or point mutations in oncocytic thyroid tumours *(55)*. Notably, oncocytic thyroid tumours are characterized by a high content of mitochondria.

1.4.5. Micro RNAs (miRNA)

Studies of microRNA in human cancer constitute a relatively new field of cancer research. It has been shown to comprise regulatory functions as oncogenes and tumour suppressive genes in different types of cancer and has also been associated with thyroid cancer. It is reported that there is up-regulation of five miRNA species, especially miR-221, miR-222 and miR-146 in PTC as compared to normal thyroid *(119)*. Following this original observation, Pallante *et al.* confirmed the involvement of miR-221 and miR-222 in addition to miR-181b *(120)*. miRNA-221 is a putative oncogene that is proposed to play a role in an early stage of PTC tumourigenesis *(119)*. In the same study, up-regulation of miR-221, -222 and -146 was shown to be associated with loss of *KIT* gene and protein expression. Interestingly PTCs with loss of *KIT* expression harboured germline single-nucleotide alterations of *KIT* that involved the recognition sequences for miR-221 and miR-222. In another follow-up study by Visone and coworkers, enforced expression of miR-221 and miR-222 resulted in reduced p27(Kip1) protein levels but not corresponding mRNA levels (*119*), and in addition gave progression to the S phase in PTC cells (121). Profiling of miRNA has also been carried out in follicular thyroid tumours, which revealed significant over-expression of miR-197 and miR-346 in FTCs as compared to FTAs and normal thyroid tissues (122).

1.4.6. Comparative genome hybridization (CGH) and loss of heterozygosity (LOH) studies

Comparative genomic hybridization (CGH) is a molecular cytogenetic method for analyzing copy number variations (CNVs) relative to ploidy level in the DNA of a test sample compared to a reference sample, without the need for culturing cells. The aim of this technique is to quickly and efficiently compare two genomic DNA samples arising from two sources, which are most often closely related, because it is suspected that they contain differences in terms of either gains or losses of either whole chromosomes or subchromosomal regions (a portion of a whole chromosome). This technique was originally developed for the evaluation of the differences between the chromosomal complements of solid tumour and normal tissue, (124) and has an improved resolution of 5-10 megabases compared to the more traditional cytogenetic analysis techniques of Giemsa banding and fluorescence in situ hybridization (FISH) which are limited by the resolution of the microscope utilized.

CGH is only able to detect unbalanced chromosomal abnormalities. This is because balanced chromosomal abnormalities such as reciprocal translocations, inversions or ring chromosomes do not affect copy number, which is what is detected by CGH technologies. CGH does, however, allow for the exploration of all 46 human chromosomes in single test and the discovery of deletions and duplications, even on the microscopic scale which may lead to the identification of candidate genes to be further explored by other cytological techniques.

Gains and losses of chromosomal regions have been described in most types of thyroid tumours including MTCs (124, 125), FTAs (126-129), FTCs (125-129) , PTCs (125, 130-135), PDTCs *(128, 136)* and ATCs (125, 128, 136-138). Taken together the detected abnormalities suggest that FTCs, PDTCs and ATCs are genetically less stable as compared

with PTCs, FTAs and MTCs *(139)*. PTC is the best studied type of thyroid tumour using CGH. It has been found that less well differentiated PTCs display significantly more abnormalities as compared to other PTCs *(131, 132)*. In addition, gains in 1p are associated with aggressive PTCs and *MUC1* (1q22) was subsequently identified to be amplified as well as over-expressed in PTCs of aggressive type *(134)*. It is reported that, the frequency of LOH is generally low in PTC *(140, 141)*, with the exception of two studies that detected LOH in 50% of cases frequently involving 4q, 5p and 7p *(139, 142)*. Cytogenetic analyses of FTA have revealed frequent rearrangements involving chromosomes 2 and 19. Furthermore, trisomies of chromosomes 4, 5, 7, 12 and 22 have been recurrently observed in this tumour group *(143, 147)*. In contrary, loss of 7q has been reported in FTC *(148-150)*. Deletions in 3p in particular the 3p21-25 region have been recurrently observed, indicating putative TSGs for FTC tumourigenesis. Loss of 22 is particularly common in FTCs, and it is associated with the widely-invasive type *(125)*. Other studies also demonstrated LOH at chromosomes 3p, 3q, 10q, 11p, 11q, 13q, 17p and 22q in FTC *(139, 151-153)*. The reported studies of CGH analyses in PDTC and ATC include a total of 33 PDTCs and 54 ATCs *(125, 128, 136)*. Of note, these studies utilized conventional CGH. Wreesmann et al. identified gains at 1p34-p36, 6p21, 9q34, 17q25 and 20q and losses at 1p11-p31, 2q32-q33, 4q11-13, 6q21 and 13q21-q31 as recurrent alterations in PDTC *(128)*. Loss at 13q was also reported in PDTC by Rodrigues *et al.*(*94*). Several alterations have been identified in this type of tumour. Hemmer *et al.* identified frequent gains of 7p22-pter, 8q22-qter and 9q34-qter in ATC, whilst Wilkens *et al.* demonstrated gains of 5p and alterations of chromosome 8 are responsible for ATC tumourigenesis *(125, 137)*. Interestingly, gains at 3p13-p14 and 11q13, and loss of 5q11-q31 were proposed to be involved in ATC progression from PDTC by Wreesmann *et al. (128)*. On the other hand, Rodrigues *et al.*, suggested that gains at 3q and 20q are associated to ATC

transformation from FTC, while losses of 7, 12q and 13q play an important role in ATC development from PDTC *(136)*.

1.4.7. Gene-specific promoter and genome-wide methylation

Aberrant methylation at the promoter region of some TSGs *i.e. RASSF1A* and *PTEN* is commonly observed not only in thyroid cancer but also in adenomas, suggesting its role is early in thyroid tumourigenesis. Methylation of several TSGs may also be associated with thyroid tumourigenesis. For example *TIMP3* (*tissue inhibitor of metalloproteinase*), *SLC5A8* (a member of the sodium solute symporter family) and *DAPK* (*calcium/calmodulin-dependent serine threonine kinase*) promoter hypermethylations were reported to be associated with poor pathological characteristics of PTC *(154)*. Recently Hoque *et al.* revealed hypermethylation of *TSHR*, *RAR*-β*2*, *CDH1* and *TGF*-β in addition to *RASSF1A*, *DAPK* and *TIMP3* in a panel of thyroid tumours *(155)*. Promoter hypermethylations were observed for thyroid hormone receptors-α and -β (TRs) mainly in FTCs and in some PTCs and FTAs *(156)*. *Fibroblast growth factor receptor 2* (*FGFR2*) is down-regulated in neoplastic thyroid cells through DNA promoter methylation *(157)*. However, little is presently known about genome-wide methylation changes in thyroid tumours. The only published study is by Galusca and colleagues who demonstrated a lower level of global methylation in thyroid cancer tissues as compared to benign tumours or adjacent normal thyroid based on analyzed using 5-methylcytidine antibodies *(158)*.

1.4.8. Expression studies

Several studies have applied cDNA microarray analyses for global gene expression profiling in thyroid tumours *(62, 133, 159-165)*. Some of the genes identified in these studies have been verified using immunohistochemistry such as *FN1* (encoding fibronectin), *LGALS3* (encoding galectin 3), and *KRT19* (encoding cytokeratin 19), suggesting that they could be developed into tools for thyroid cancer diagnostics *(166, 167)*. Gene expression profiles are

distinctly different in FTC as compared to PTC. Over-expression of *Cbp/p300- interacting transactivator* (*CITED1*), *claudin-10* (*CLDN10*), and *insulin-like growth factor binding protein 6* (*IGFBP6*) was seen in PTC, while e.g. *caveolin-1* (*CAV1*) and *-2* (*CAV2*) were expressed at similar levels in the two tumour types . Using a similar approach Fryknäs *et al.* observed significant under-expression of the *four and a half LIM domains 1* (*FHL1*) gene in FTCs as compared to FTAs *(168)*. Molecular profiling studies have also been applied with the aim to clarify molecular events in thyroid tumour dedifferentiation. Montero-Conde *et al.* found that genes related to the MAP kinase and TGF-β-signaling pathways, focal adhesion, cell motility, activation of actin polymerization and cell cycle were up-regulated in ATC as compared to WDTCs *(169)*. Similarly another study also revealed that up-regulation of a set of genes involved in cell cycle progression and chromosome segregation is a unique feature of ATC *(165)*.

1.5. Rationale of the Study

As mentioned above, the wide divergence in clinical behavior of thyroid carcinoma is poorly predicted by currently used clinico-pathological factors for diagnosis and prognosis. Hence, the different molecular alterations in various thyroid carcinomas that have been recently identified are being explored clinically. Despite extensive research to date, especially in genetic alterations, the scientific community has not been successful in translating biomarkers into useful clinical tools and thus avail the full clinical potential for patients presenting indeterminate thyroid nodules.

Attempts to improve diagnostic accuracy in the cases of uncertain or suspected cytological tests in thyroid nodule-FNAB samples with the addition of *BRAF* mutation, detection of *RAS, RET/PTC,* and *PAX8/PPARγ* mutations, are being made. This is expected to improve cancer diagnosis. *BRAF,* can also be used as a tool for tumour prognostication based on the recent evidences and in clinical practice. The genes, coding the signaling cascade proteins (*RET,*

RAS, BRAF, PI3K, PTEN, AKT), are mutated or aberrantly expressed in thyroid cancer derived from follicular thyroid cell. Genetic and epigenetic alternations also contribute in consequence of malignant follicular cell transformation. The clinical implications of these molecular alterations include their utility in diagnostic evaluation, staging, prognosis and targeted treatment. Thus molecular analysis of thyroid cancer promises to increase our understanding of its biologic behavior and is expected to have further impact on its clinical management.

RMC has one of the largest registries of thyroid cancer patients in South East Asia. Approximately 500 new cases are being diagnosed every year as thyroid cancer. However the type and prevalence of various mutations in thyroid cancer patients remain so far unidentified in Indian population. It is known that different geographic areas and different genetic background amongst races may contribute to different frequencies and type of mutations as well as their role in the molecular pathogenesis of thyroid cancer.

Therefore the project aims at:

- To study the prevalence of *BRAF* ^{V600E} mutation and its implication as diagnostic and prognostic tool in thyroid carcinoma
- To study the prevalence of *BRAF* mutation in poorly differentiated and tall cell variant of thyroid carcinoma
- Detection of *RET/PTC* and *PAX8-PPARγ* rearrangements and their correlation with *BRAF* mutation and clinico-pathological parameters in thyroid carcinoma
- study the prevalence of *RET* (*Exon 10,11,13,14,16*) gene mutation in Medullary thyroid carcinoma
- study the prevalence of *NRAS, HRAS* and *KRAS (Exon 1, 2)* gene point mutation in thyroid cancer patients of Indian origin

Chapter 2

Prevalence of Point mutations in Follicular Cell Derived Thyroid Carcinomas

2.1 Activating Mutation in *BRAF* Gene in Thyroid Carcinoma

2.1.1 Introduction:

2.1.1.1 Papillary Thyroid Carcinoma:

Papillary thyroid cancer (PTC) is the most common histological type of thyroid malignancy and the rising incidence of thyroid cancer is mainly attributed to the increased diagnosis of PTC, particularly the small PTC *(170, 171)* [Fig 2.1.1]. PTCs generally have a good prognosis after appropriate treatment including surgical procedure and radioiodine therapy *(172, 173)*.

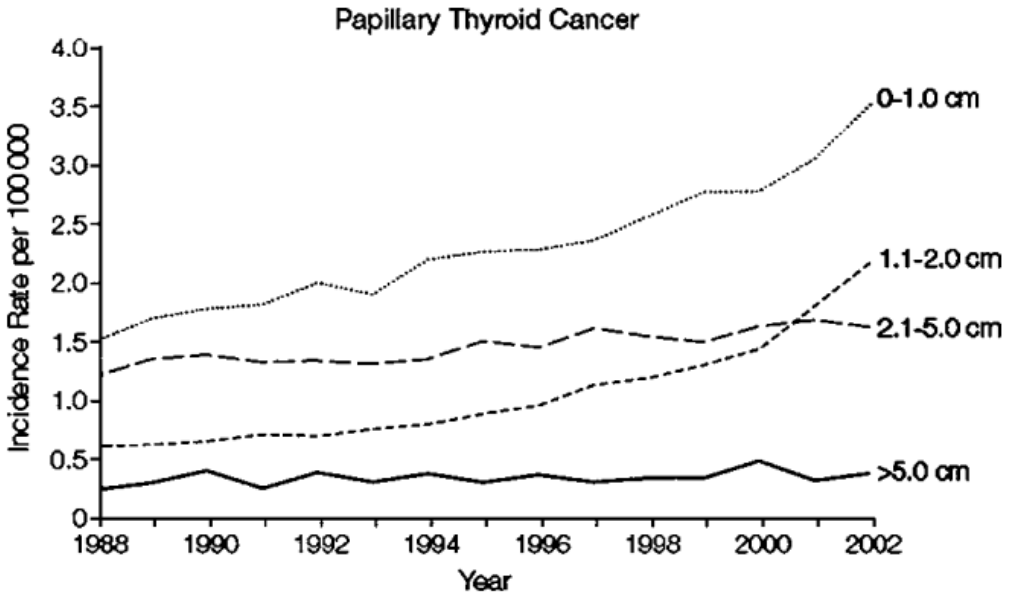


Fig 2.1.1: Increasing incidence of papillary thyroid carcinoma; Trends in different size of tumours [Source: Davies and Welch *(175)*]

However, the recurrence rate of differentiated thyroid cancer and death rate are now reported to be as high as 30%, and 8% respectively, at 30 years of follow-up, after initial treatment *(174, 175)*. During the past decade, understanding of genetic alternations in thyroid cancer has rapidly expanded. These improvements in understanding the development of thyroid cancer have offered novel diagnostic tools and therapeutic strategies.

Similar to other cancer types, the progression and dedifferentiation of thyroid cancer involves a number of genetic alterations including two distinct molecular mechanisms: point mutation or chromosomal rearrangement *(176)*. Most mutations in thyroid cancer involve the MAPK and PI3K–AKT signaling pathways. *BRAF* is a serine-threonine kinase that is translocated to the cell membrane after being bound and activated by *RAS*, which results in the phosphorylation and activation of mitogen activated protein kinase (MAPK) and other downstream targets of MAPK signaling pathway *(177, 178)*. According to our present studies, *BRAF* mutation exclusively exists in PTC and PTC-derived anaplastic thyroid cancer (ATC). It has not been found in other histological types of thyroid cancer such as follicular thyroid cancer (FTC) and medullar thyroid cancer (MTC) *(83, 179)*. The *BRAF* ^{V600} mutation [a valine to glutamic acid mutation at position 600] is found in more than 50% of all thyroid malignances *(180)*. In our study, we focused on the association of *BRAF* mutation with clinic-pathological characteristics and potential use of the *BRAF* mutation as a diagnostic marker and therapeutic target of thyroid cancer.

2.1.1.2 *BRAF* ^{V600E} mutation related molecular events in PTC

The *BRAF* gene (official name: v-raf murine sarcoma viral oncogene homolog B1) encodes B type RAF protein, one of the components of MAP kinase pathway, as mentioned before. There are 3 isoforms of *RAF* serine-threonine kinase: *A RAF, B RAF*, and *C RAF (181)*. Although all the RAF isoforms have an ability of MEK activation, BRAF protein is the most

effective MEK activator, characterized by different distribution & expression in various tissues *(182)*.

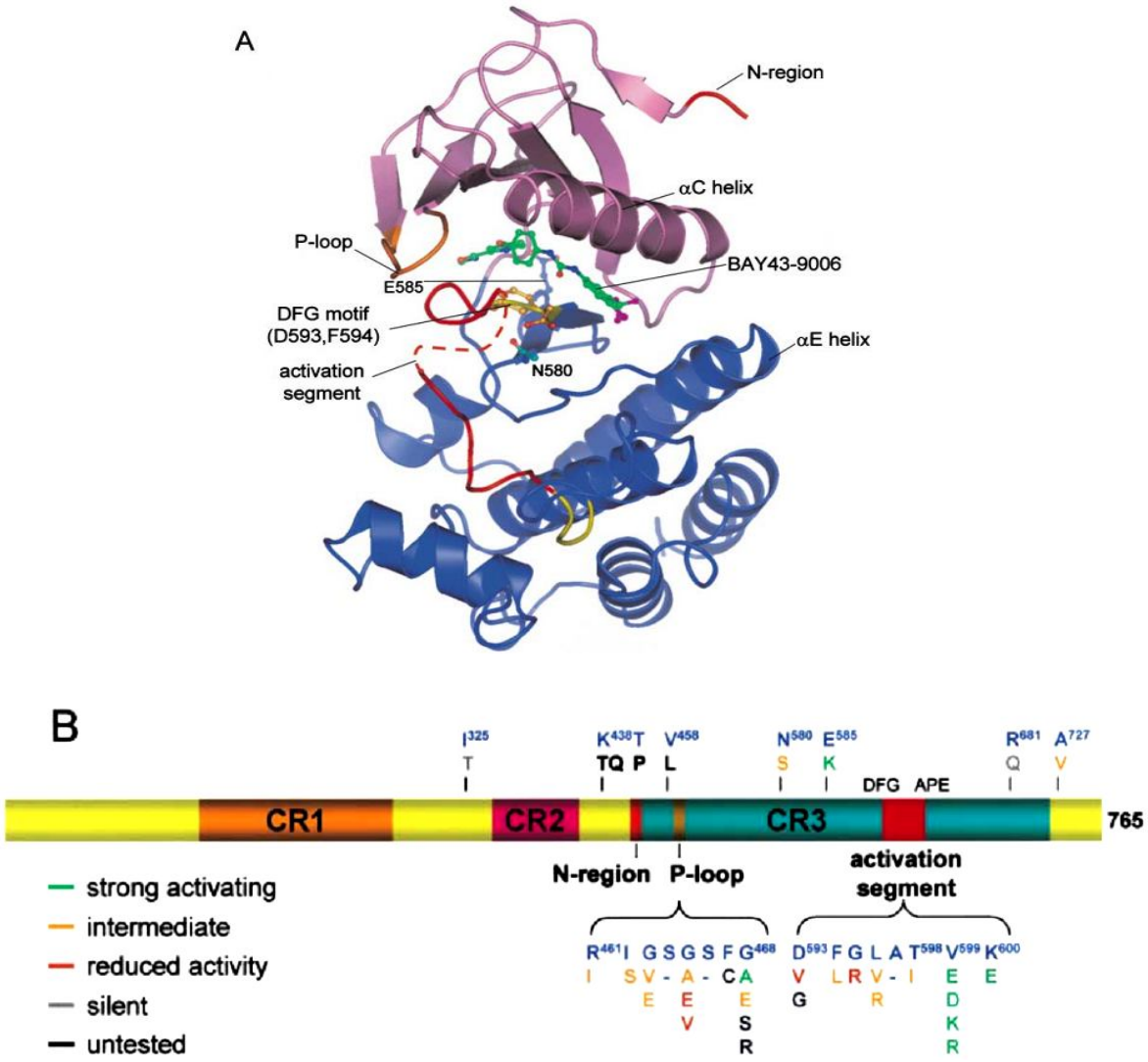


Figure 2.1.2: (A) Ribbons diagram of Wild Type B-RAF kinase do-main. The positions of Asp593 and Phe594 of the DFG motif, Asn580 of the catalytic loop, and Glu585 are shown, DFG and APE motif in yellow, rest of activation segment and the N region are in red. N lobe is in magenta, C lobe in marine, and P loop in orange. Residues 600–611 of the activation loop are disordered (dashed lines). **(B)** Schematic of B-RAF primary structure, showing functional domains and position of 32 observed cancer associated mutants of B-RAF. [Source: PyMOL (http://www.pymol.org)]

BRAF is a regulated signal transduction serine-threonine kinase with 766 amino acids. As explained in Fig: 2.1.2, it consists of three conserved domains: CR1, a RAS –GTP binding

domain; CR2, a serine rich hinge region and a catalytic kinase domain, CR3, that activates the downstream proteins by phosphorylation *(183, 184)*. There are 2 known loci for human *BRAF* gene; *BRAF1 – 7q34*, responsible for coding the functional product & *BRAF2 – Xq13*, it constitutes the inactive pseudo gene, of which the gene located on chromosome 7, is the most potent activator of the MAP kinase pathway *(185)* [Fig: 2.1.3].

The most common genetic alterations in PTCs include *BRAF* mutation, *RAS* mutation, and *RET/PTC* rearrangement, which are mainly involved in the *RAS*/*BRAF*/*MAPK* signal pathway. Interestingly, these molecular alterations are exclusive in PTC patients, suggesting that each of them alone is sufficient for malignant transformation of thyroid cells. *BRAF* ^{V600E} mutation strongly increases *BRAF* kinase activity by eliciting ERK1/2 phosphorylation which is 480 fold higher than wild type *BRAF (186)*. The markedly increased ERK1/2 phosphorylation in *BRAF*^{V600E} mutation is mainly attributed to a negatively charged residue adjacent to the phosphorylation site at T598 and mimics phosphorylation at Thr598 and Ser601 residues *(82)*.

Activation of MAPK pathway by *BRAF* ^{V600E} was believed to play a dominant role in the development and progression of thyroid cancer. Effectors of MAPK signaling pathway represent an early molecular event in PTC by regulating a number of genes related to cell proliferation, differentiation and survival. Gene expression analyses using DNA microarrays showed that different transcriptional profiles were associated with *BRAF*, *RET / PTC* and *RAS* mutation groups in human PTCs *(187)*. The differential expression profiles between these genetic alternations may explain why *BRAF* mutation has more noticeable correlations with poor clinic-pathological features compared with *RET /PTC* and *RAS* mutation. The mutant *BRAF* can stimulate constitutive signaling which bypasses the need for extracellular growth factors. Subsequently phosphorylation of downstream MER1/2 and ERK1/2 leads to the expression of a number of specific genes involved in cell proliferation, differentiation,

proliferation, differential, survival, tumourigenesis, and even the microenvironment promoting the process of epithelial-mesenchymal transition (EMT) *(188)*. The mutant *BRAF*/*MAPK* pathway is illustrated in Figure 2.1.4.

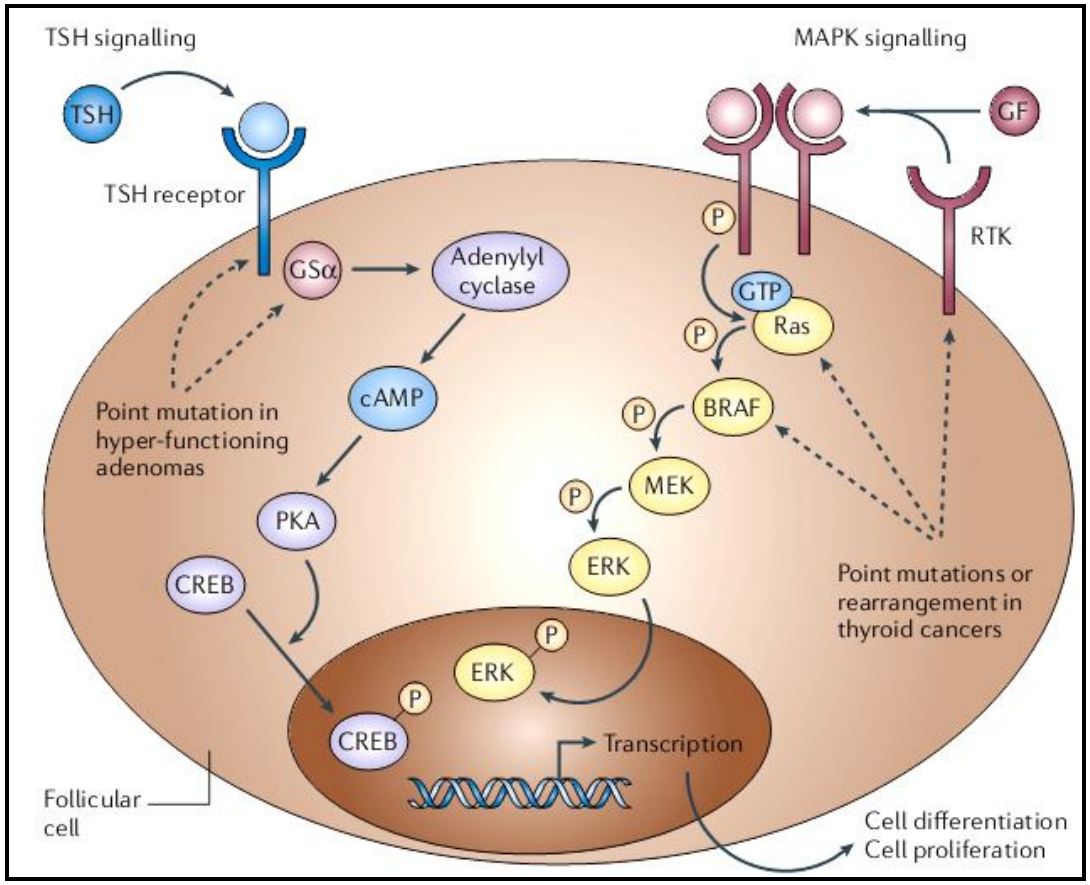


Figure 2.1.3: MAPK signaling pathways in Thyroid. Growth factors (GF) induce receptor-tyrosine kinase (RTK) dimerization, resulting in phosphorylation of specific tyrosine residues within the cytoplasmic tail which activates *RAS* by catalyzing the replacement of GDP with GTP. *RAS* activates the kinase activity of *BRAF* and its downstream signaling cascade. *BRAF* phosphorylates the mitogen-activated protein kinase (MAPK) kinase (MEK), which phosphorylates and activates extracellular-signal-regulated kinase (ERK). Activated ERK migrates to the nucleus where it phosphorylates and activates various transcription factors that are involved in cell proliferation and differentiation, such as MYC and ELK1.
Thyroid follicular cells upon binding to TSH produce cyclic AMP (cAMP) which stimulates the protein kinase A (PKA). PKA in turn phosphorylates cytoplasmic and nuclear target proteins.
(Source: Nature Reviews- Cancer, Vol. 6, April 2006)

It has been demonstrated that methylation of several tumour suppressor genes including tissue inhibitor of metalloproteinase3 (TIMP3), SLC5A8, death associated protein kinase

(DAPK), and retinoic acid receptor b2 (RARb2) was closely associated with *BRAF* mutation in PTC *(154)*. The silencing of these tumour suppressor genes represented an important molecular mechanism in *BRAF* mutation induced progression and invasiveness of PTC. Another study showed that PTCs harbouring *BRAF* mutation were significantly associated with the expression of both MMP2 and MMP9, which was associated with a higher more frequency of extra-thyroidal invasion with respect to MMP negative PTCs *(189)*. The process of absorption and accumulation of radioiodine relies on the sodium iodide symporter (NIS) in the basal membrane that transports iodide into the cells from the blood stream. Impairment of NIS expression and other iodide metabolizing genes including TPO, TG, and pendrin was found to be associated with *BRAF* mutation in PTCs *(190)*. One explanation may be that the gene silencing by methylation regulated by *BRAF* mutation, which resulted into loss of radioiodine sensitivity and increased aggressiveness of PTC.

2.1.1.3 Association of *BRAF* mutation with clinic-pathological characteristics of PTC

$BRAF^{V600E}$ mutation in thyroid cancer has been proved to be associated with high risk clinic-pathological characteristics, tumour recurrence and reduced sensitivity of radioiodine therapy. Conventional factors that demonstrate the high risk clinic-pathological characteristics included increased age, male gender, larger tumour size, extra-thyroidal invasion, local lymph node metastasis, distant metastasis, and advanced disease stages. Recent studies have shown significant correlations of *BRAF* mutation with the reliable prognostic predictors such as extra-thyroidal invasion, lymph nodal metastasis, and advanced TNM stage *(191, 192)*. A meta-analysis revealed that PTC patients harbouring $BRAF^{V600E}$ mutation achieved a 1.5 to 2.1fold increase in extra-thyroidal extension, lymph node metastasis, and advanced TNM stages compared with those holding wildtype *BRAF (193)*. However, a number of studies have also demonstrated that there was no significant association between *BRAF* mutation and poor clinic-pathological factors *(194, 195)*.

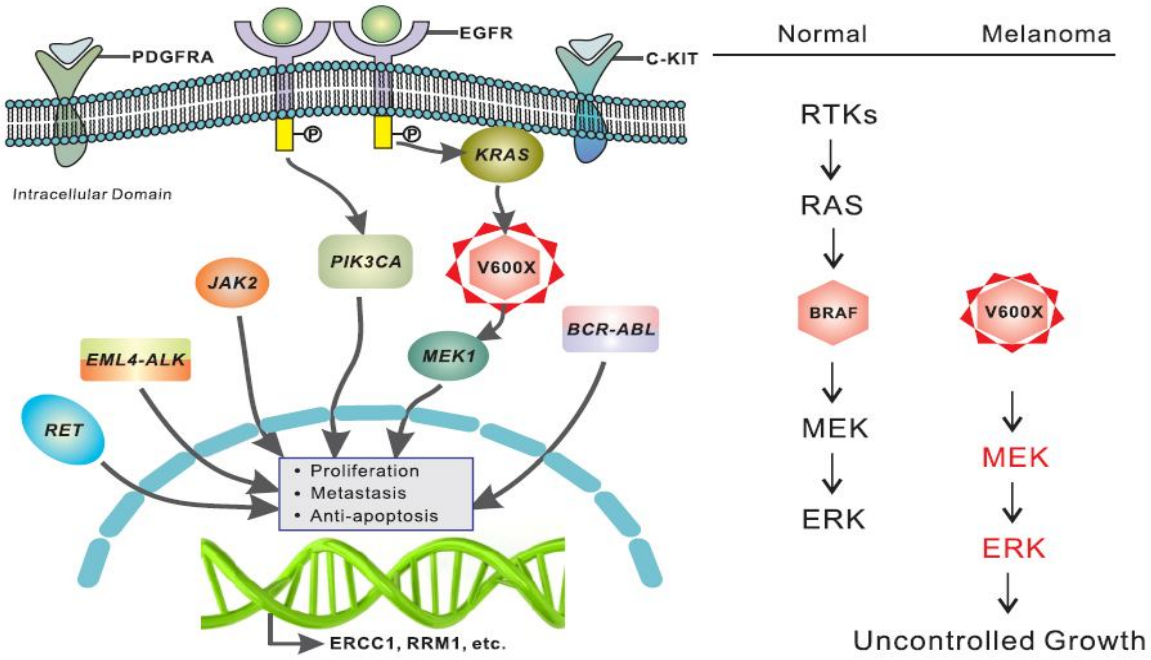


Fig 2.1.4: Normal *BRAF* is activated by interaction with *RAS*. In $BRAF^{V600E}$ mutation proteins have high unregulated kinase activity that impels cell proliferation and tumour growth. (Source: http://www.biosb.com/braf)

The association of *BRAF* mutation with PTC recurrence was displayed in a majority of the studies including multivariate analysis with adjustment for all the known prognostic factors, even in patients with early tumour stages I and II *(191, 196, 197)*. However, a recently a study suggested that $BRAF^{V600E}$ mutation was not associated with the recurrence of PTC after initial treatment with total thyroidectomy and radioiodine remnant ablation *(198, 196)*. The rather aggressive initial treatment in this study may have contributed to the absence of significant recurrence in patients with *BRAF* mutation. It has also been indicated in yet another study that $BRAF^{V600E}$ mutation correlated strongly with radioiodine resistance in PTC patients due to the reduced expression of Sodium Iodide Symporter and lost capacity for iodine uptake *(199)*. However, the impairment of iodide metabolism in PTC patients

harbouring *BRAF* mutation can be compensated by carrying out radioiodine ablation therapy with an aggressive dose.

2.1.1.5 Detection of $BRAF^{V600E}$ mutation as a molecular marker in thyroid cancer

Diagnosis by fine needle aspiration biopsy (FNAB), the gold standard for preoperative evaluation of thyroid lesions, has recently been used in conjunction with molecular tests to improve the accuracy of diagnosis from cytology and has been used in 15 – 20% of thyroid nodules with indeterminate outcomes on FNABs *(200)*. It is feasible to look for molecular markers including *BRAF*, *RAS*, *RET/PTC* and PAX8/PPAR γ, which may be helpful to increase the diagnostic accuracy with FNAB *(201, 202)*.

BRAF mutational analysis, in particular, has improved the sensitivity of diagnosis of PTC in FNAB specimens is well documented *(203)*. It has been proposed that preoperative knowledge of *BRAF* mutation of the thyroid nodules may help surgeons make proper decision regarding the extent of operation, such as subtotal thyroidectomy versus total thyroidectomy, and neck dissection versus no neck dissection. Hence, appropriate surgical plan before surgery may seem to be possible especially for patients at early stages and reduce the postoperative complications accordingly. Compared with clinico-pathological evaluation using traditional indicators, testing of *BRAF* mutation seems to be more useful to identify the risk of tumour recurrence *(191,204)*.

2.1.1.6 Targeting *BRAF* signaling pathway as therapeutic target to treat thyroid cancer

Several molecule inhibitors of *BRAF* have been developed including Sorafenib, PLX4032, RAF265, PLX4720, and XL281 with different selectivity *(205)*. Indeed, encouraging results with the *BRAF* inhibitors Sorafenib and PLX4032 were recently reported in clinical trials with malignant melanoma which has a high prevalence of *BRAF* mutations. These drugs showed markedly inhibition of cell proliferation, survival, motility, and invasion *in vivo* and

in vitro. PLX4032, a compound which selectively targets *BRAF* ^{V600E}, can effectively inhibit the proliferation of PTC cell lines bearing *BRAF* mutation *(206, 207)*. However, incomplete inhibition of ERK activation by PLX4032 was also revealed in comparison with the MEK inhibitor PD0325901 which completely inhibited ERK activation. The disabled feedback mechanisms and wildtype *BRAF* proteins may contribute to the incomplete inhibition of ERK activation. Moreover, recent study suggested that *BRAF* activates nuclear factor κB (NF κB) and this pathway is MEK independent *(208)*.

Although increasing clinical trials using these selective pathway inhibitors have shown promising results in patients with tumours harbouring *BRAF* ^{V600E} mutations, acquired resistance to these agents is an emerging problem. The clinical effectiveness and safety of the inhibitors tested was generally limited, raising the question on the effectiveness of inhibiting only the MAPK pathway to target resistant and aggressive tumours with *BRAF* mutations.

2.1.2 Aims and Objectives:

From the above information it is apparent that sporadic point mutations in BRAF proto-oncogene in several human cancers, including thyroid carcinoma, results in constitutive activation of downstream MAP kinase pathway. Most prevalent among them is the transversion mutation that occurs at nucleotide position 1799 of *BRAF* gene, resulting in a valine to glutamic acid substitution at residue 600 (*BRAF*^{V600E}) *(180, 209)*.

Several studies have been carried out to see if a correlation exists between *BRAF*^{V600E} mutation and clinico-pathological features of PTC. However, the results have been controversial. This may be due to the sample sizes, inclusion of different PTC variants in the study, and variations due to different geographic areas *(194, 195, 210)*.

It is known that uncertain prevalence might bias the potential usefulness of *BRAF* mutation as a diagnostic and prognostic molecular marker for PTC *(179, 204, 211)*. Hence, it is

mandatory to build up the knowledge of prevalence of *BRAF* mutation and it's relation with the clinical behavior of PTC in different cohorts from various geographical regions *(209, 212)*.

The overall incidence of thyroid cancer in India is 34% with 80% of it being PTC *(1)*. However the type and prevalence of the *BRAF* mutations in PTC patients remains so far unidentified in the Indian population *(209, 212)*. Hence, the aim of the present study was to establish the prevalence of $BARF^{V600E}$ mutation and to define association of this genetic alteration with clinico-pathological characteristics of PTC in Indian subcontinental population. For this purpose, 140 sporadic thyroid carcinoma patients with different histological variants were included in the study. The mutational analysis of the *BRAF* gene was performed by an assay based on MASA, Mutector assay, SSCP or by PCR and direct sequencing.

2.1.3 Materials and Methods:

2.1.3.1 Tumour Tissue Specimens:

Frozen thyroid tissues from thyroid cancer patients who had attended the clinic between 2002 and 2006 were retrieved from the Tissue Repository of Tata Memorial Hospital, Mumbai, India, for analysis of *BRAF* gene mutation at codon 600. A total of 140 specimens with adequate clinical and pathological information were retrospectively studied. Tissue specimens included 86 PTC, 14 FTC, 8 FTA, 16 MTC and 16 benign hyperplasias. Acquisition of the tissue specimens was approved by the Scientific Review Committee of Tata Memorial Hospital. Experiments were performed in accordance with regulations of the hospital's Human Ethics Committee. The clinical information was retrieved retrospectively from patients' medical records, pathology reports, and subsequent clinical visits. Histological diagnosis was reconfirmed by an experienced pathologist after hematoxylin and eosin staining of sections. Patients were staged using the tumour-node-metastases (TNM) system

and classified according to the presence of extra-thyroidal extension, cervical nodes and distant metastases.

2.1.3.2 Cell Lines:

Five thyroid tumour cell lines were used in this study (Table 2.2.1). Follicular carcinoma (WRO), papillary carcinoma (NPA), and anaplastic carcinoma (ARO and FRO) cell lines were procured from Dr. Giorgio Stassi's laboratory ((Universita Degli Studi Di Palermo, Palermo, Italy) which had been originally developed by Dr. Guy J. F. Juillard at the University of California at Los Angeles. Medullary thyroid carcinoma cell line (TT) was received from National Centre for Cell Sciences (NCCS), Pune, India. All these thyroid carcinoma cell lines were grown in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal calf serum at 37ºC and 5% CO_2 *(179)*.

2.1.3.3 Molecular Analysis:

2.1.3.3 A. Genomic DNA Extraction:

Nucleic acid extraction from frozen tissues, cell lines and normal peripheral blood mononuclear cells (PBMNC) was carried out using QIAamp® DNA Mini Kit (Qiagen, Germany) with some modifications. All samples were subjected to digestion in digestion buffer (Tris HCl 100mM, EDTA 1mM, 1% SDS) containing 1mg/ml of proteinase K at 60°C for 12 hrs. Concentration of extracted DNA was quantified by measurement of OD at 260 nm in Nanodrop Reader (Nanodrop Technologies, ND1000, U.S.A).One hundred nanograms of genomic DNA was used as template for detection of mutation.

2.1.3.3 B. PCR Amplification:

Genomic DNA (100 ng) was used as template to amplify 215 bp fragment of *BRAF* gene exon 15. Sequences of the forward 5'TCATAATGCTTGCTCTGATAGGA3' and reverse 5'GGCCAAAATTTAATCAGTGGA3' primers were based on flanking introns *(179)*. PCR

reaction was set with an initial denaturation of 3 min at 94°C followed by thirty five cycles where each cycle consisted of denaturation for 30 s at 94°C, annealing for 45 s at 55°C, extension for 45 s at 72°C and a final extension step for 10 min at 72°C. The NPA and ARO cell lines were used as positive controls for *BRAF* T1799A mutation, and WRO cell line and PBMNC were used as controls for wild type *BRAF* gene.

2.1.3.3 C. Sequencing:

BRAF gene exon 15 amplicons generated from tumour tissue, cell lines and PBMNCs were subjected to automated DNA sequencing in an ABI PRISM 37718 DNA Sequencer (ABI, USA). Each amplicon was sequenced in both directions using Big Dye Terminator Version.3.1 Cycle Sequencing kit (ABI, USA) and above mentioned forward and reverse primers respectively. All amplicons were cleaned by QIAquick gel extraction kit (Qiagen, Germany) before being used as template. Cycle sequencing reaction and PCR program was followed as per manufacturer's protocol. Sequences were compared by the BLAST program: www.ncbi.nlm.nih.gov/BLAST.

2.1.3.3 D. Mutant allele specific amplification (MASA):

MASA was carried out with two forward primers, one complimentary to wild type sequence, and the other having mutant allele specific nucleotide base substitutions at the 3'end (5'TAG GTG ATT TTG GTC TAG CTA CAG T3'and 5'GGT GAT TTT GGT CTA GCT ACA AA 3') and the common reverse primer used earlier. Each primer was designed to amplify the wild type allele of *BRAF* T1799A and transversion mutation respectively *(211)*. Amplification reactions were performed in a PTC1148 M J Thermal Cycler (MJ BioRad, U.S.A), following an initial denaturation of 2 min at 94°C and subsequent denaturation for 30 s at 94°C, annealing for 45 s at 52°C and extension for 45 s at 72°C for 35 cycles (40 cycles for negative samples for verification). Amplicons were separated on 2 % agarose gel and

visualized by ethidium bromide staining. All samples were examined twice for the conformation of *BRAF* mutation.

2.1.3.3 E. Single strand conformation polymorphism (SSCP):

Exon 15 amplicons of *BRAF* gene were diluted 1:1 in denaturing solution (0.25% Bromo-phenol blue and 95% formamide and 0.1M NaOH) before boiling for 3 min in boiling water bath and rapid chilling on ice to prevent reannealing of single strand products. Electrophoresis was carried out in the MacrokinS Electrophoresis Unit (Techno Source, India) with Fotodyne DNA Seq. System, Model 4200, USA, at 9°C, 120 V, 30 mA for 6 hrs. Gels were stained with silver nitrate *(213)*.

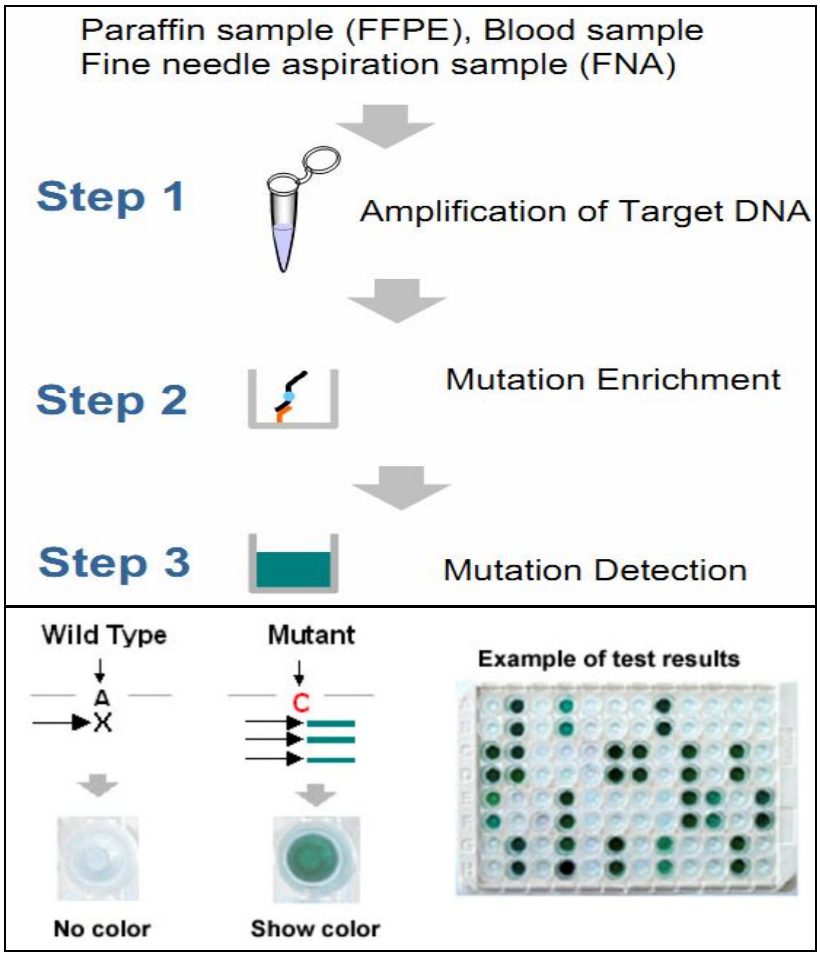


Fig 2.1.5: The Mutector™ *BRAF*^{V600E} detection kit is a highly sensitive colorimetric mutation detection test, designed to accurately identify *BRAF*^{V600E} mutations in codon T1799. (Source: www.trimgen.com/products/kras)

2.1.3.3 F. Mutector™ Assay:

Mutector assay was also used to detect *BRAF* mutation at nucleotide position 1799. The Mutector assay is designed to detect point mutations of known DNA sequence variation. For this, a detection primer was used (provided in kit) which allows primer extension in cases of mutant amplicons and prevents primer extension for wild type template. If primer extension does not occur, labeled nucleotides are not incorporated, and a color reaction is not observed.

When the target base is mutated (e.g., T→A point mutation at *BRAF* T1799), primer extension continues and a strong color reaction is observed. The Mutector assay is highly sensitive and can detect as little as 1% of mutant DNA from a mixed sample. The assay was performed using 10 μL of PCR products (215 bp fragment) according to the manufacturer's instructions (TrimGen, Sparks, MD) *(214)*. Positive and negative controls were used as mentioned for PCR analysis.

2.1.3.4 Statistical analysis:

Patients' clinico-pathological parameters and mutation status were tabulated using mean and frequency. Correlation between *BRAF* mutation and clinico-pathological parameters of the patients was determined by χ^2 test and non-parametric two-tailed Fisher test and multivariate logistic regression analysis. A univariate as well as multivariate analysis using logistic regression was performed to assess the effect of clinico-pathological variables on lymph node metastasis. All analyses were performed using Sigma Stat version 3.5. $P < 0.05$ was considered as statistically significant for two tails *(215)* .

2.1.4 Results:

2.1.4.1 *BRAF* mutation in thyroid cancer cell lines:

Direct sequencing of exon 15 amplicons obtained from DNA of 5 human thyroid cancer cell line (Fig 2.1.6) was confirmatory for their respective genotypes. As seen in the electropherogram, NPA was homozygous for *BRAF* T1799A (V600E) mutation, while ARO

and FRO cell lines were heterozygous. This missense mutation was absent in WRO and TT cell lines which are homozygous for wild type allele. All these cell lines (Table 2.1.1) were used as controls for further experiments.

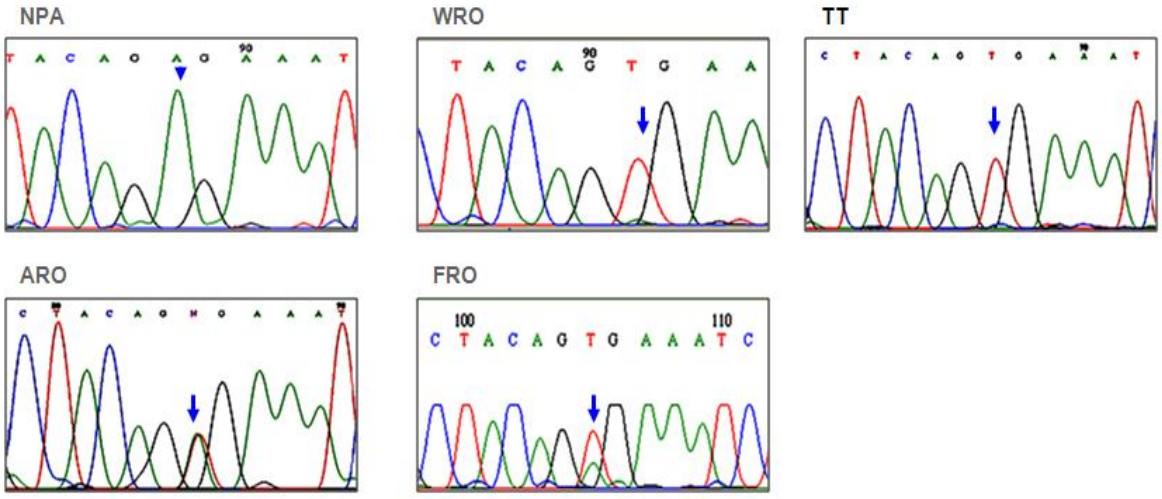


Figure 2.1.6: ***BRAF*^{V600E}** mutation in thyroid cancer cell lines as demonstrated by DNA sequencing. The sequences show the wild type *BRAF* gene in WRO & TT, homozygous *BRAF* mutation T1799A (V600E) in NPA and heterozygous *BRAF* mutation T1799A in ARO & FRO.

TABLE 2.1.1: *BRAF*^{V600E} mutation in thyroid carcinoma cell lines

Cell line	Type	Metastasis	*BRAF* ^{V600E}
WRO	FTC	Yes	-
NPA	PTC	Unknown	+
ARO	ATC	Yes	+
FRO	ATC	Yes	+
TT	MTC	Unknown	-

2.1.4.2 *BRAF* mutation in thyroid tumour samples:

BRAF^{V600E} mutation was determined primarily by MASA assay, Mutector assay, DNA sequencing and SSCP in 25 samples. Results obtained by all three techniques were

comparable. Mutation detected in all the positive samples by MASA (Fig 2.1.7) involved a T>A transversion at nucleotide 1799 (V600E) and was heterozygous as shown by DNA sequencing (Fig 2.1.8 B). Mutector assay (Fig 2.1.8 A) showed high sensitivity for the mutation and allowed quantitative detection of percent mutant in template DNA in samples (data not shown). However, it is however not cost effective for screening large number of samples by this method. Thus, MASAPCR which is specific, reproducible and also economic was found to be most suitable for screening all of the 140 tumours along with adjacent normal tissue. Fifty percent of all the samples were randomly selected for DNA sequencing. SSCP analysis (Fig 2.1.7) corroborated with the above findings but was found to be inconsistent among samples, since SSCP does not have the requisite sensitivity, when the mutated DNA content is low.

In summary, *BRAF* T1799A (V600E) mutation was found in 46 out of 86 PTC patients (53.4%) included in the study. Percent incidence of this mutation varied among pathological subtypes of PTC (Table 2.1.2). *BRAF* gene mutation was much more common in the conventional PTC (38 out of 62; 61.3%) than in the follicular variants of PTC (2 out of 17; 11.7%). None of the 54 non-PTC thyroid tumours including 8 FTA, 14 FTCs, 16 MTCs and 16 benign samples showed any mutation at this nucleotide position in exon 15. Also, normal thyroid tissue samples from the surroundings of *BRAF* mutation positive malignant thyroid areas showed no mutations of *BRAF* thereby suggesting that the mutations were somatically acquired.

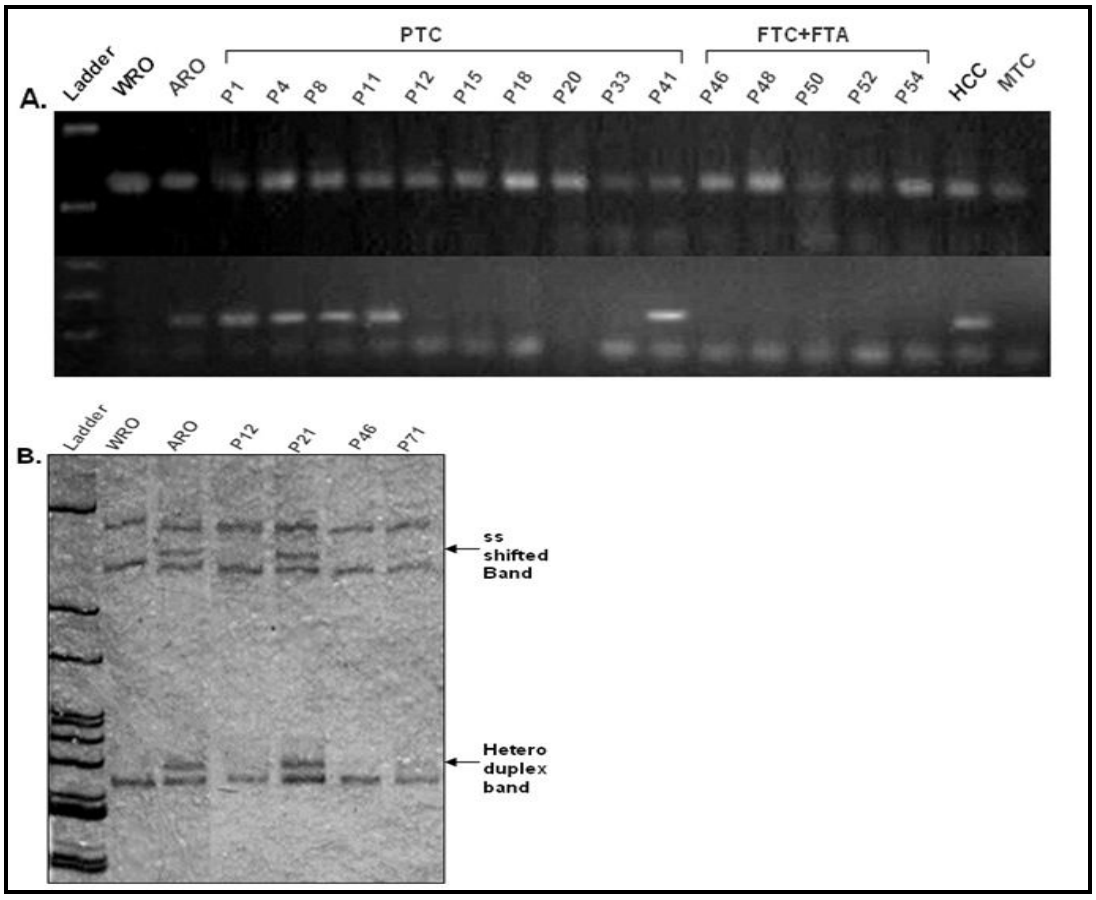


Figure 2.1.7: *BRAF* mutation in Thyroid Tumours. **(A)** Mutant allele specific amplification (MASA) analysis of *BRAF* mutation. Genomic DNA extracted from tumour tissue was analyzed for *BRAF* mutation by MASAPCR as described in Materials and Methods. PCR products were analyzed in a 2% agarose gel. Genomic DNA extracted from WRO and ARO Thyroid cancer cell lines were used as negative and positive control respectively.**(B)** Single strand conformational polymorphism (SSCP) pattern in thyroid tumours showing WRO (*BRAF*T1799WT), ARO (*BRAF* T1799A Transversion mutation) and DNA samples from thyroid cancer patients. Samples P12 and P46 show wild type *BRAF* whereas samples P21 and P71 exhibit mutated *BRAF*. Notice the abnormal single strand and heteroduplex pattern in the mutated samples (arrows).

Table 2.1.2: Prevalence of *BRAF*^{V600E} mutation in 140 thyroid neoplasia patients

Histology	Total	*BRAF*^{V600E}			
		Positive		Negative	
		N	%	N	%
Papillary Carcinoma	86	46	53.4	40	46.6
Classical Variant	62	38	61.3	24	38.7
Poorly Differentiated (PDTC)	2	2	100	0	0
Foll. Var. of PTC (FVPTC)	17	2	11.7	15	88.2
Tall Cell Variant	3	3	100	0	
Hurthle Cell Carcinoma (HCC)	1	1		0	
Diffuse Sclerosing variant	1	0		1	
Follicular Carcinoma	14	0		14	
Follicular Adenoma	8	0		8	
Medullary Carcinoma	16	0		16	
Benign hyperplasia	16	0		16	

2.1.4.3 Correlation with clinico-pathological parameters:

The association between *BRAF* mutation and various clinico-pathological parameters were assessed in 86 patients with PTC (Table2.1. 3). By univariate analysis, *BRAF* mutation status showed significant association with conventional type of PTC (P<0.011), TNM Stage (P<0.005), extra-thyroidal extension (P<0.005) and lymph node metastasis (P<0.005). There was no significant correlation between *BRAF* mutation and sex, age or tumour size at diagnosis. A multivariate analysis was performed with the same clinico-pathological parameters. Only extra-thyroidal extension (odds ratio = 9.1; 95% confidence interval, 2.75–30.4, P<0.005) and lymph node metastasis (odds ratio = 3.5; 95% confidence interval, 1.12–11, P<0.031) were correlated with *BRAF* mutation with and without stepwise multiple logistic regression method.

All the tumours were divided into morphological subtypes, wherein conventional (classical) variant of PTC was the largest group. Correlation of *BRAF* mutation with the same parameters was sought within this group. However, no significant correlation was found either by univariate or by multivariate analysis.

To understand the prognostic significance of clinico-pathologic factors in determining lymph node metastasis, linear and multiple regression analyses were performed. In univariate analysis, tumour size, tumour stage, ETE and *BRAF* mutation were significant predictive factors for lymph node metastasis (Table 4). But in multivariate analysis tumour stage and *BRAF* mutation were the only significant prognostic factor for lymph node metastasis (odds ratio = 12.2; 95% confidence interval, 4.1–35.6, P <0.001; and odds ratio = 4.9; 95% confidence interval, 1.5–15.5, P <0.006; respectively) (Table 2.1.4).

In summary, our results predict that *BRAF* gene mutation of PTC is correlated with lymph node metastasis and ETE by both univariate and multivariate analysis; but it is not an independent risk indicator within the classical subtype, as observed by other investigators. Tumour stage and *BRAF* gene mutation were the only significant prognostic factors for lymph node metastasis in PTC. However, *BRAF* mutation status is not independent from PTC subtypes in predicting extra-thyroid extension and lymph node metastasis *(215)*.

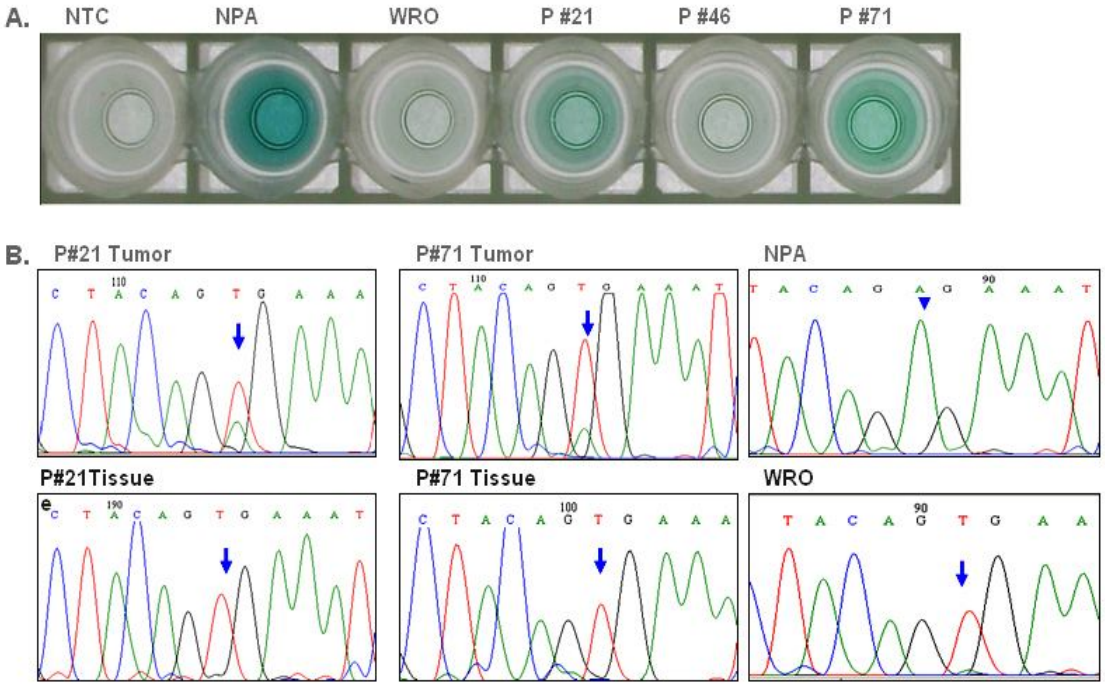


Figure 2.1.8: *BRAF* mutation in Thyroid Tumours. (**A**) Results of Mutector assay by *BRAF* codon 600 mutations detection kit; TrimGen®, Sparks, MD. Wells with color reaction (*green*) represent samples positive for T1799A *BRAF* mutation. (NTC) is no template control, (NPA) the Papillary thyroid cancer cell line served as the positive control and (WRO) the Follicular thyroid cancer cell line served as the negative control. The representative positive and negative thyroid tumour samples were P#21, P#71, and P#46, respectively. (**B**) The corresponding sequence chromatographs of tumour P#21,tumour P#71 and NPA, with *BRAF* T1799A mutation and normal tissue P#21, P#71 and WRO with wild type *BRAF* T1799.

TABLE 2.1.3: Correlation between *BRAF*^{V600E} mutation and clinico-pathological parameters in papillary thyroid carcinoma

	BRAF^{V600E}				χ^2 test	Multivariate Analysis	
	Positive		Negative		*P* €	*P*	Odds ratio(95%CI)
	n †	%	n	%			
Tumour Stage (PTC)					< 0.005	0.381	1.4 (0.613.5)
T I	2/18	11.1	16	88.9			
T II	10/22	45.4	12	54.6			
T IIIIV	34/46	73.9	12	26.1			
Gender (PTC)					<0.65	0.073	0.31(0.081.1)
Male	16/32	50.0	16	50.0			
Female	30/54	55.5	24	44.5			
Age(PTC)					<0.085	0.11	2.6 (0.88.5)
≥45yr	24/38	63.1	14	36.8			
<45yr	21/48	43.7	27	56.2			
Node Metastasis (PTC)					<0.005	0.031	3.5 (1.111.4)
Yes	35/47	74.4	12	25.6			
No	11/39	28.2	28	71.8			
Tumour size (mm)					<0.068	0.94	0.96 (0.352.6)
range, mean ± SD	3/12	25.0	9	75.0			
<10 mm	32/52	61.5	20	38.5			
10–40 mm	11/22	50.0	11	50.5			
>40 mm							
Distant Metastasis	2/3	66.6	1	33.4			
Extra-thyroidal Extension					<0.005	<0.005	9.1 (2.730.4)
Yes	33/41	80.5	8	19.5			
No	13/45	28.9	32	72.1			
Tumour Type					0.011	0.098	3.1 (0.8212.2)
Conventional	40/65	61.5	25	38.4			
NonConventional	6/21	28.5	15	7.1			

€ Clinico-pathological parameters are compared by $\chi 2$ test

† Data are presented as no./total no. of patients, with percentages in parentheses

*Age and Tumour size were assessed as continuous variables

2.1.4.4 Pathological findings:

Among the 86 heterogeneous entities of PTC, the conventional subtype was found to predominate over other types. In general, *BRAF* mutation status was associated with certain clinical features like: older patients, larger tumours, solid tumours, higher rate of node metastasis and extra-thyroidal extension (ETE). The presence of *BRAF* mutation also correlated well with pathologic features like papillary patterns, characteristic nuclear features, intra-thyroidal invasion, sclerotic stroma etc. It was seen more commonly in conventional types and all cases of tall cell variants (3/3). *BRAF* negative finding was associated more frequently with younger patients, smaller tumours, cystic tumours, lower rate of lymph node metastasis, ETE, nonpapillary patterns like follicular and solid pattern circumscribed border, subtle nuclear features and lack of sclerotic stroma. This group included mainly FVPTC and other tumour types.

Both the cases of poorly differentiated thyroid carcinoma (PDTC), we found, were of large cell type. Both patients were male. On histological examination, the tumour showed areas of PTC with vacant nuclei and nuclear grooves (Fig. 2.1.9). *BRAF* mutation was present in both patients, indicating the progression of PTC towards PDTC.

In the three cases with distant metastasis, all were having lymph node metastasis and ETE and one of them was PDTC. Two out of three distant metastasis patients were *BRAF* mutated as reported by others too *(216)*.

However, in summarizing statistical analysis and morphological findings, our results indicate that *BRAF* mutational status, though associated strongly with distinct clinical, pathologic and biologic variables, it is not an independent prognostic indicator ‘per se’ in PTC. Morphologic typing appears to be essential as a prognostic indicator in PTC.

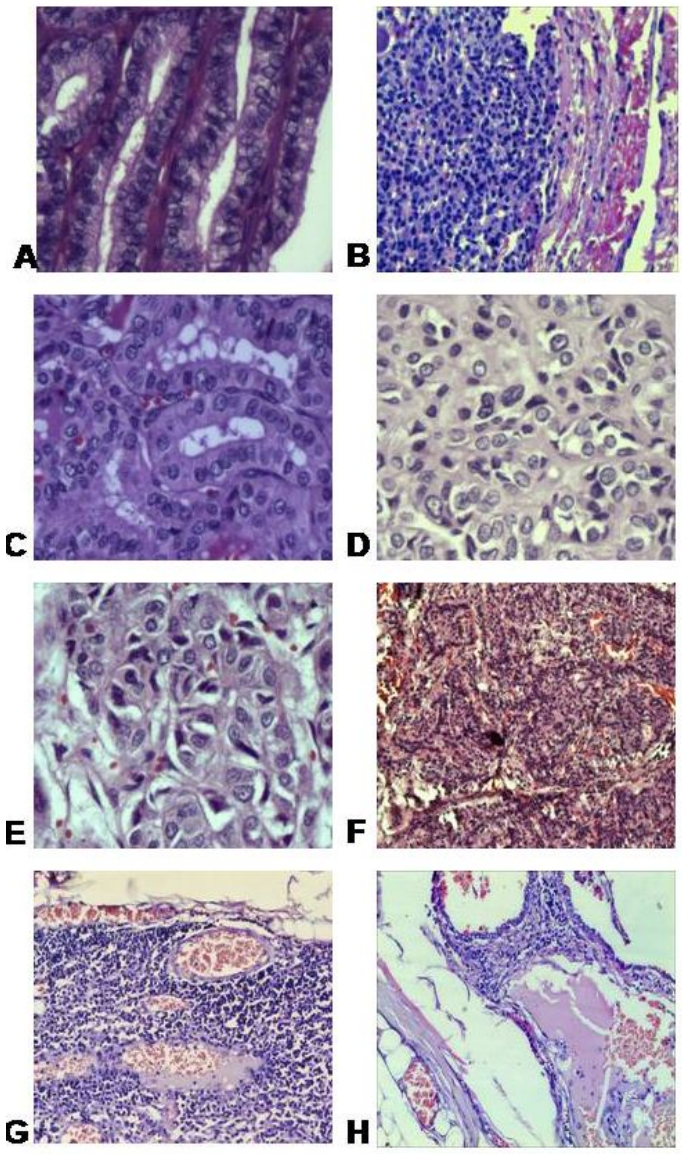


Figure 2.1.9: Pathological variants of PTC. **(A)** Conventional PTC with characteristic nuclear feature **(B)** FVPTC with circumscribed border (capsular) **(C)** Tall cell variant of PTC with compactly arranged papillae and dense eosinophilic cytoplasm (D) Poorly differentiated PTC with vacant nucleus and nuclear grooves (E) Hurthle cell carcinoma (F) Diffuse sclerosing variant of PTC (G) Lymph node metastasis in PTC (H) ETE with cystic changes in PTC

TABLE 2.1.4: Prognostic Factors for Lymph Node Metastasis

	Lymph Node Metastasis		Univariate Analysis		Multivariate Analysis	
	Yes	**No**	**P**	**Odds Ratio**	**P**	**Odds Ratio**
Age*			0.81	1.1 (0.42.67)	0.22	0.97 (0.941.0)
Gender			0.82	1.1 (0.52.7)	0.72	1.2 (0.43.6)
Female	29	25				
Male	18	14				
Tumour Size*			0.037	1.3 (1.01.7)	0.50	1.3 (1.01.9)
TNM Stage			0.001	12.2 (4.135.6)	0.001	12.2 (4.135.6)
I	0	18				
II	1	21				
III & IV	46	0				
ETE			0.001	5.6 (2.114.3)	0.052	3.1 (0.999.8)
Yes	31	10				
No	16	29				
BRAF ^{V600E}			0.001	7.4 (2.819.3)	0.006	4.9 (1.515.5)
Positive	35	12				
Negative	11	28				

* Age and Tumour size were assessed as continuous variables for both univariate and multivariate analysis

2.1.5 Discussion:

Since the initial discovery of *BRAF* mutation in human cancers *(209)*, there have been more than 40 mutations identified in the *BRAF* gene. Amongst these mutations the T1799A point mutation is the most common and accounts for more than 90% of all the mutations found in the *BRAF* gene *(217)*.

BRAF^{V600E} mutation was reported in thyroid carcinoma initially by Kimura et al. 2003 *(179)* and since then significant progress has been made in understanding its tumourigenic role and clinical importance in this disease *(210, 218)*. It has been shown that *BRAF* plays critical role

as intermediate of ERK phosphorylation in MAPK pathway. This pathway acts in parallel with TSH receptor cAMP-PKA-CREB pathway for subsequent cellular proliferation and differentiation *(55)*. There is a crosstalk between these two pathways, either agonistic or antagonistic, depending on the cell type *(219)*.

This activating mutation has been specifically reported in PTC with a frequency ranging from 29–83% in several thyroid tumour cohorts depending on the epidemiological factors, heterogeneity of the histological variants, or the age group analyzed *(220, 221)*. Recently Mathur et al. have reported a much higher rate (88%) of *BRAF* mutation in PTC which increased significantly over a 15 year period at the authors' institution *(216)*. The findings suggested that a higher rate of *BRAF* mutation in papillary thyroid cancer may contribute to the increasing incidence of thyroid cancer. Xing et al. 2005 have shown that this mutation is associated with a poorer clinico-pathological outcome and is a novel independent molecular prognostic marker in the risk evaluation of thyroid cancer *(191)*.

In Mumbai, 3-4% of the cancer patients are reported to suffer from thyroid neoplasia *(1)*. To the best of our knowledge there is no published data on the prevalence of *BRAF* mutation in thyroid cancer in patients from the Indian sub continent.. In the present study, we report the frequency of occurrence of *BRAF* mutation in sporadic PTCs, mainly in the classic variant of PTC, for the first time in our patients and confirm that this mutation is strongly associated with the papillary growth pattern.

In the present study, 140 patients of thyroid carcinoma were included from different parts of the country. $BRAF^{V600E}$ mutation was found in 46 out of 86 PTC patients (53.4%), in comparison with observations from other Asian countries, Japan 36 %, Taiwan 46.6 % and Ukraine 22.9 % as quoted by Fugazzola et al. *(222)*. We did not find the concerned mutation in the adjacent normal thyroid tissues of those patients with $BRAF^{V600E}$ positive tumour samples, which is suggestive of its somatic origin *(204, 209)*.

The distribution of *BRAF* mutation in PTC showed a clear subtype related pattern. We found the significant prevalence of *BRAF* mutation in classical PTC (64%).Whereas in follicular variant of PTC (FVPTC) only 11% harboured *BRAF* mutation. This percentage of prevalence correlated well with the average prevalence of 60% and 12% incidence of *BRAF* in classical PTC and FVPTC as observed from the nine reports summarized in the review by Xing M, *(83)*. Also, all the tall cell variants, which were more aggressive in nature, harboured *BRAF* mutation as expected *(83, 223)*.

In our study, both the poorly differentiated carcinoma (PDTC) samples with large cell component were positive for $BRAF^{V600E}$. The tumours contained areas of PTC with vacant nuclei and nuclear grooves. Similar finding were reported by Nikiforova et.al, 2003 and Soares et al. 2004 *(224, 225)*. According to our observation two out of three distant metastasis cases were harbouring *BRAF* mutation with one of them being PDTC. In the study reported by Fugazzola et.al, none of the 10 poorly differentiated tumours were positive for *BRAF* mutation, indicating the absence of *BRAF* mutation in PDTC when not associated with a well differentiated component; which is in contrary to our observation *(216)*. Our finding is also supported by authors who have reported *BRAF* mutations only in PTC and PTC derived anaplastic or poorly differentiated tumours. The possibility that *BRAF* mutation reduces the risk of tumour progression was a matter of debate a decade ago *(224, 225)*, which as suggested by Puxeddu et al. 2004, it might be related to differences in cancer selection or to the low number of cases studied in each tumour cohort, due to unavailability of samples *(226)*. However, presently, the role of BRAF in tumourigenesis is accepted *(189)*.

As far as the correlation of *BRAF* mutation and clinical features of PTC patients are concerned, we did not find significant correlation between *BRAF* mutation and gender, age, tumour size at diagnosis. Whereas we found significant correlation of *BRAF* mutation with nodal metastasis ($p < 0.005$), extra thyroidal invasion ($p < 0.005$) and tumour stage ($p < 0.005$)

which is in agreement with the observation reported by Frasca et al. 2008 in a series of 323 PTC patients indicating that the presence of *BRAF* mutation in PTC is associated with aggressive tumour behavior *(189)*. In the multivariate analysis, *BRAF* mutation correlated only with ETE and lymph node metastasis. In another study on 500 cases of PTC in a homogenous Italian cohort from a single institution Lupi et al. demonstrated a strong association of *BRAF* mutation with extra-thyroidal invasion, lymph node metastasis and advanced tumour stages *(227)*. However, we could find no correlation of *BRAF* with any of these clinico-pathological parameters, when performed within the classical or follicular subtype of PTC. In our consecutive series of 86 patients, tumour stage was another prognostic factor, other than *BRAF* mutation, for lymph node metastasis. Similar to our finding, one large scale study on 410 PTC patients reported by Cheng S et al. indicated that *BRAF* mutation cannot be considered as independent risk determinant within a particular subtypes PTC *(215)*. Hence, this association is still controversial as it is observed in some studies, but not in other studies. Different factors such as number of study subjects in a cohort, different recruitment and follow up procedures, tumour classification may have been contributing factors towards making many of these studies inconclusive *(218)*. Our results predict that *BRAF* gene mutation of PTC is correlated with lymph node metastasis and ETE by both univariate and multivariate analysis; but it is not an independent risk indicator within the classical subtype.

We have not included studies on other genetic alterations such as *RET/PTC* and *RAS* in this context, but done subsequently to study translocation and rearrangements, and is the subject of a later Chapter in the Thesis. However it has been reported that $BRAF^{V600E}$ occurs mutually exclusively and its activation may be responsible for progression to classic PTC *(179, 223, 227, 228)*. According to our study all the PDTC, Hurthle cell, tall cell variant, and most of the distant metastatic patients were *BRAF* positive, which suggests that this mutation may be

involved in thyroid cancer progression to poorly differentiated and aggressive phenotypes also as reported by Michels et al. *(229)*. The results from the transgenic mouse studies also revealed that *BRAF* mutation initiated development of PTC and its transition to anaplastic thyroid cancer *(230)*. Consistent with other observations, we did not find any patient with FTC, FTA, MTC or benign hyperplasia, positive for *BRAF* mutation *(216, 224)*.

In conclusion, this study undertaken to determine the prevalence of $BRAF^{V600E}$ in the thyroid cancer patients from the Indian population and the clinico-pathological parameters associated with the cancer. We found 53% of PTCs harboured *BRAF* mutation which is as expected and concurs with literature reports ranging from (29 – 83%). This genetic event is predominantly associated with the classic variant of PTC also in Indian subjects and correlated significantly with aggressive features among all the clinico-pathological parameters that we studied. However, statistical analysis and morphological findings, in combination suggests that *BRAF* is not an independent prognostic indicator 'per se' in PTC.

Thus our findings, along with those reported in the literature; suggest that morphologic typing in combination with *BRAF* mutation status appears to be better prognostic indicator in PTC. Relevantly, considering that the prevalence is >50% in the Indian cohort, *BRAF* gene can be a promising target for small molecular inhibitors for better prognosis of radioiodine refractory thyroid carcinoma patients, which is also reported by King et al. *(231)*.

2.2 *BRAF* Mutation in Tall Cell Variant of Thyroid Carcinoma

2.2.1 Introduction:

2.2.1.2 Tall cell Variant of Papillary Thyroid CA:

Within papillary thyroid carcinoma, numerous histopathological subtypes have been described. Among them, follicular, macro-follicular, Pseudo-Warthin, clear cell have an indolent course and better prognosis *(232)*. Though there is some controversy between tumour morphology and prognosis, subtypes like tall cell variant (TCV) and columnar cell variant have been associated with more aggressive clinical course. Hence, at present there are endeavors to clearly define the correlation between prognostic characteristics and histological subtypes of thyroid carcinoma.

Table 2.2.1: Proposed Criteria for the Diagnosis of PTC TCV

- Tumour composed of 50% tall cells
- Tall cell height at least twice its width
- Eosinophilic tall cell cytoplasm
- Nuclear features characteristic of PTC

TCV was first described in 1976 by Hawk et al. *(232)*. Reported incidence of TCV range from 4% to 17% of total PTC cases *(233)*. It is generally characterized by abundant eosinophilic cytoplasm, numerous papillae, classical nuclear pattern of PTC and height at least twice or thrice their width *(229)*.

Diagnosis of TCV is quite challenging (Table 2.2.1) *(52, 234-236)*. Their nuclear stratification and exaggerated intranuclear inclusions are often noted (Fig: 2.2.1). TCV has also a low nuclear cytoplasmic ratio and an eosinophilic granular cytoplasm due to an abundance of mitochondria demonstrated (Fig 2.2.1) *(237)*. Although many classical PTC

have some tall cells, a significant proportion of the tumour needs to be composed of tall cells to label the carcinoma as TCV. Unfortunately, there is disagreement with regard to the threshold needed to classify a tumour as TCV. Indeed, the cutoff values range from 30% to 70% according to different publications *(233, 235, 238)*. Probably due to these facts, only 2 (1.4%) of 140 patients studied in our population could be classified as TCV.

Mutations in *BRAF* proto-oncogene and its close association of $BRAF^{V600E}$ with extra-thyroidal extension and lymph node metastasis in papillary thyroid carcinoma has been discussed in previous chapter and reported by others *(239)*. Our findings have also been published *(240)*. Though yet unclear, there may be a correlation between the aggressiveness of TCV and *BRAF* gene mutation *(84, 241)*. There are literature reports on the presence of a rare *BRAF* mutation (*BRAF* I582M) in classical PTC histology leads to the replacement of amino acid isoleucine to methionine *(242)*. However, there are no available reports of this mutation in TCV.

Influence of *RET-PTC* rearrangement and *PAX8-PPARγ* translocation, which play a critical role in the oncogenic transformation of classical and follicular variant of PTC, are also equivocal in TCV patients *(189)*. Studies on different ethnic populations of the world indicate elevated expression of MUC1, Cyclin D1, Galectin 3, HBME 1, Cytokeratin 19 in PTC patients at the transcriptional and translational level as reported by Cheng et al., using high throughput proteomic analysis *(243)*.

Among the two patients with TCV, one was a female patient who had recurrences more than 14 times and will be documented as a case repot considering BRAF and other genetic markers. In this patient, we have investigated the close relationship between these prognostic and diagnostic markers in TCV of PTC in the context of multiple recurrences. This study

shows the need for studying more cases to understand the association of these markers with this rare histotype.

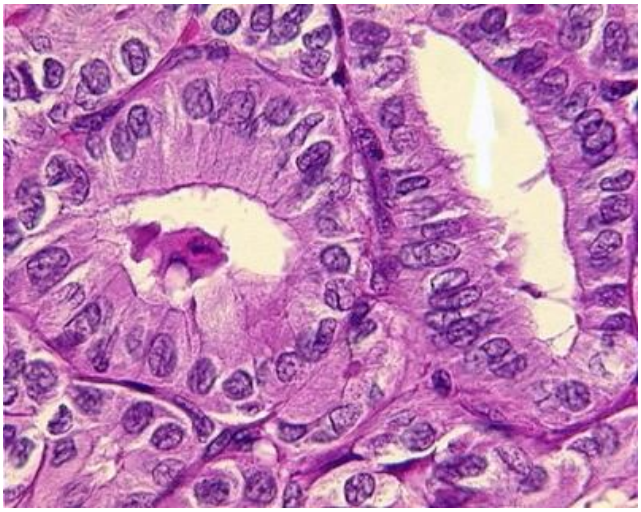


Fig 2.2.1: Example of Tall cell variant (TCV) of papillary carcinoma. Note the abundance of granular acidophilic cytoplasm with oncocyte like features. (Source: Cheng Z. Liu, et.al April 30, 2003 University of Oklahoma Health Science Center)

2.2.2 Case report: *BRAF* ^{V600E} Mutation Along with a Rare *BRAF* ^{I582M} Mutation Documented in Tall Cell Variant of Papillary Thyroid Carcinoma with Multiple Recurrences

A 46 year old female with a clinical diagnosis of papillary thyroid carcinoma had undergone near total thyroidectomy ten years previously. Macroscopic examination of the specimen showed a 33gm gland with the left lobe measuring 5.2 x 2.3 x 3.3 cm and the right lobe measuring 5.5 x 2.5 x 2.3 cm**.** Within three months of the first surgery, a second operation was carried out to remove the growth over the tracheal surface. Histopathological examination proved this to be metastatic lymph node of PTC**.** Five years after this surgery there was a development of large palpable mass on the thyroid bed. Excision biopsy of the mass was suggestive of metastatic deposits of papillary carcinoma of thyroid in lymph nodes**.** In view of already two surgeries undertaken previously to remove nodal recurrences, she was considered for empirical radioiodine therapy and was treated with 155 mCi ^{131}I. There was

minimal response and, hence, was undertaken for the neck dissection for the recurrent mass (Fig 2.2.3). Histopathology denoted the presence of soft tissue of PTC with left side neck node metastasis. A distinct tall cell pattern was observed for the first time in the course of this malignancy from initial diagnosis. Two years following this, another recurrence was detected, necessitating the surgical removal of left sided cervical lymph node. It was found to be metastatic PTC of tall cell pattern**.** Subsequently, she presented with neck nodal recurrences over six years, which necessitated surgical removal. None of the times, there was any radioiodine concentration observed in the tumour recurrences and, hence, ^{131}I therapy was not administered. Whole body survey with [^{18}F]FDG-PET had shown relatively avid uptake in the neck recurrences as can be expected in this case (Fig 2.2.4); on the last occasion the standardized uptake value (SUVmax) calculated was 10.3. Interestingly, distant metastases were not documented in the previous FDG-PET studies. The thyroglobulin values were elevated during each of the recurrences and ranged from 23 ng/ml to 340 ng/ml.

Following the last surgery, the patient was also treated with external radiotherapy to the neck in view of multiple recurrences. Very recently, she presented with elevated Tg (24ng/ml). Latest FDG PET-CT study revealed multiple FDG avid nodules in both the lungs with largest nodule documented as 1.7mm (SUV max 38.1).

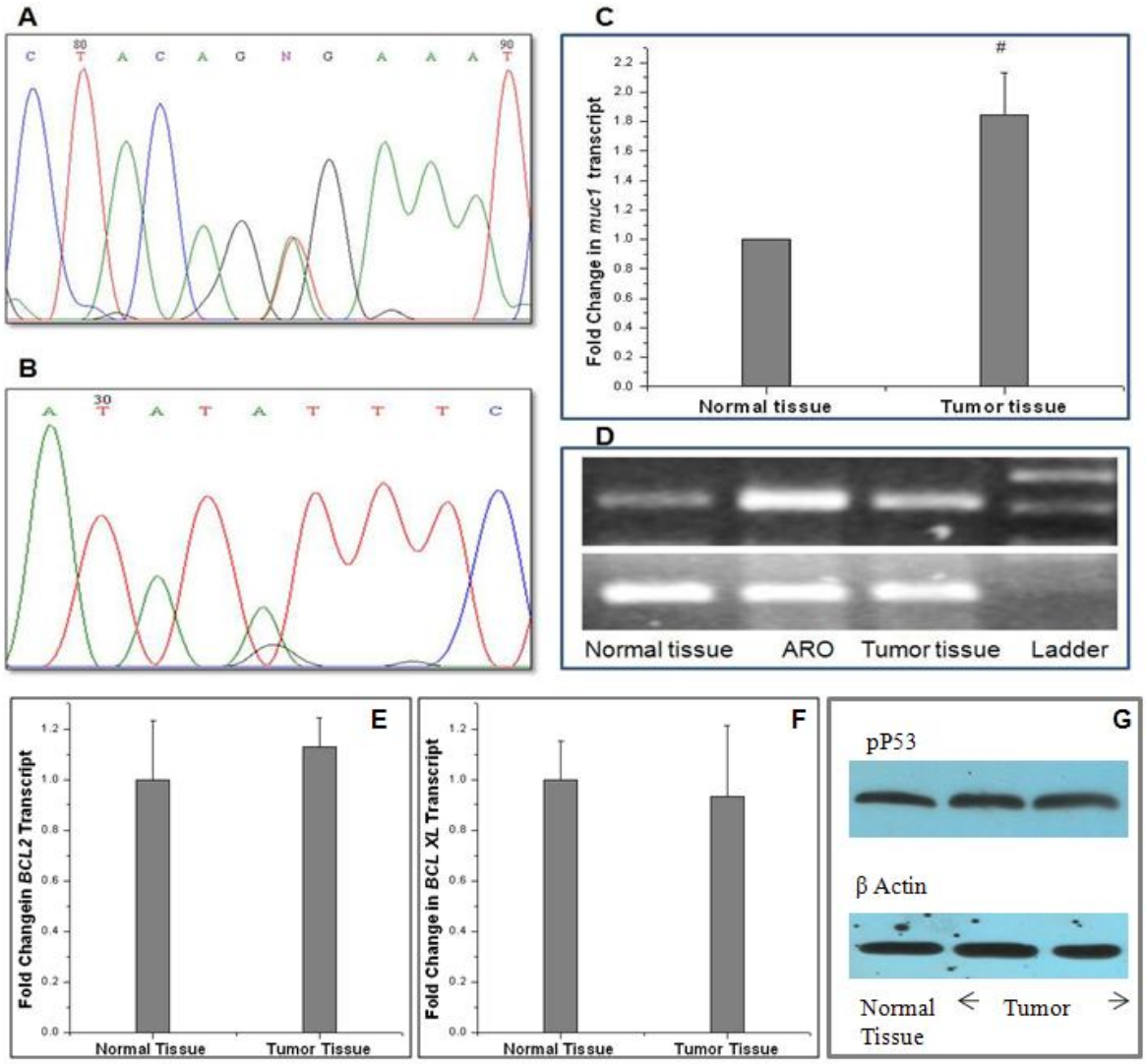


Fig 2.2.2: *BRAF* mutation in Thyroid Tumours. **(A)** Sequence chromatograph of tumour showing heterozygous *BRAF* T1799A mutation. Genomic DNA extracted from tumour tissue was amplified for *BRAF* Exon 15 and analyzed for *BRAF* mutation by dideoxy sequencing. **(B)** *BRAF* I582M point mutation observed in the same tissue specimen. **(C) Real time PCR:** The graph shows relative mRNA expression corrected for total mRNA using the housekeeping βactin gene. Two fold increase on MUC1 level in metastatic carcinoma tissue with respect to adjacent normal tissue. (D) Semiquantitative RT PCR: *muc1* gene (upper) expressing in negative control (normal tissue), positive control (ARO cell line) and Thyroid tumour tissue with their respective β Actin genes (lower). **(E) and (F) Real time PCR:** The diagrams show that there were no significant difference in relative mRNA expression of BCL2 and BCL XL gene in metastatic carcinoma tissue with respect to adjacent normal tissue. **(G) Western Blot**: pP53 protein level (upper) was identical in both normal tissue and tumour tissue with respective β Actin level (lower).

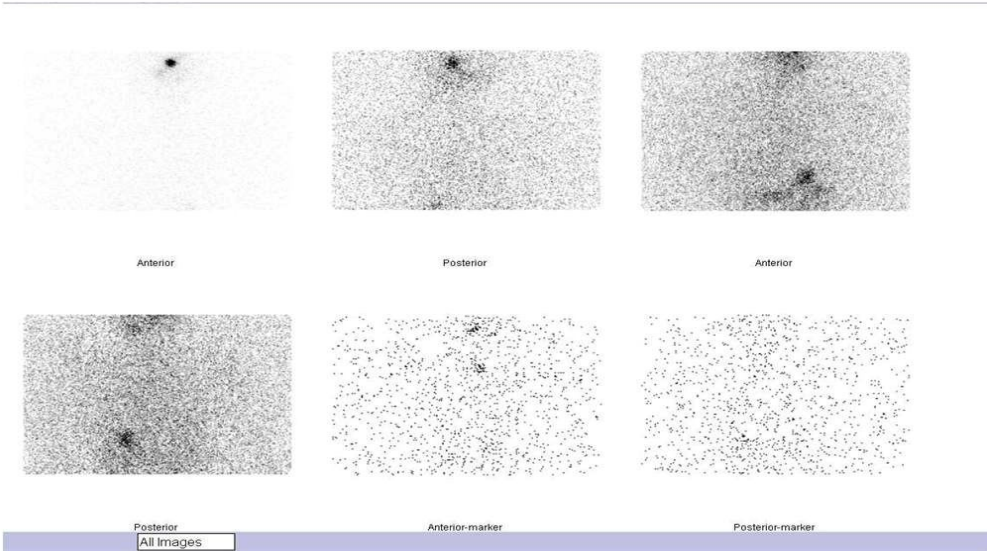


Fig 2.2.3: ^{131}I Scan: High count neck and chest view of the I131 whole body scan during the last recurrence undertaken 72 hours after oral administration of 148 MBq I^{131} demonstrates no abnormal focus in the neck and chest, implying the palpable neck nodes are non iodine concentrating (a feature of dedifferentiation in the thyroid carcinoma).

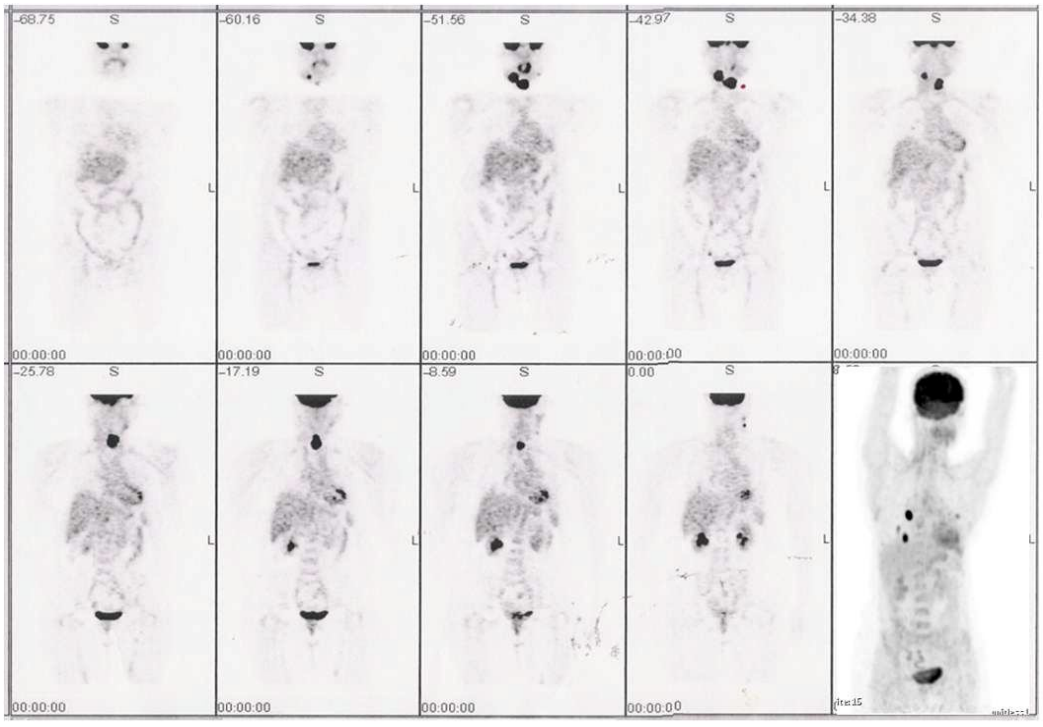


Fig 2.2.4: FDGPET Scan: Whole body FDGPET (coronal slices) during the time of last recurrence) undertaken 60 minutes after injection of 370 MBq [^{18}F]FDG demonstrates multiple FDG avid neck nodes (SUVmax 10.3). The rest of the whole body survey was unremarkable.

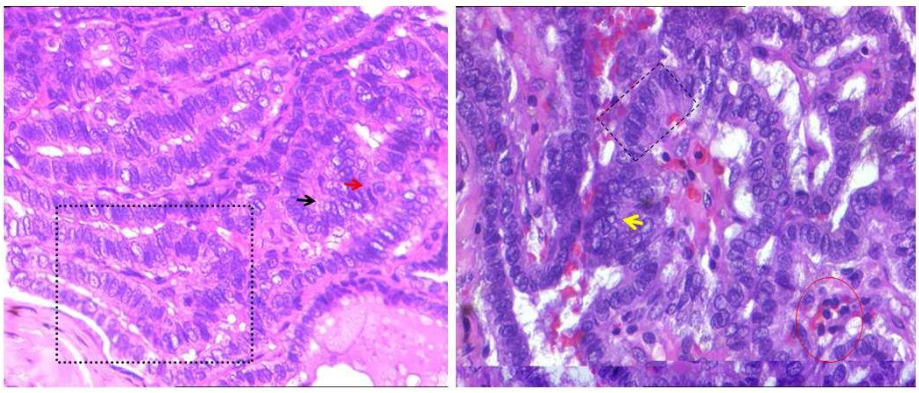


Fig 2.2.5: H & E sections (200X): Tall cell variant of PTC. (A) Note compactly arranged finger like projections. The cells are tall with dense eosinophilic cytoplasm. Overlapping nuclei oriented along the longitudinal axis, with nuclear pseudoinclusion, are the characteristic features. (B) H&E section (400X) shows papillary projections covered by tall cells. Characteristic nuclear features are better appreciated in this figure.

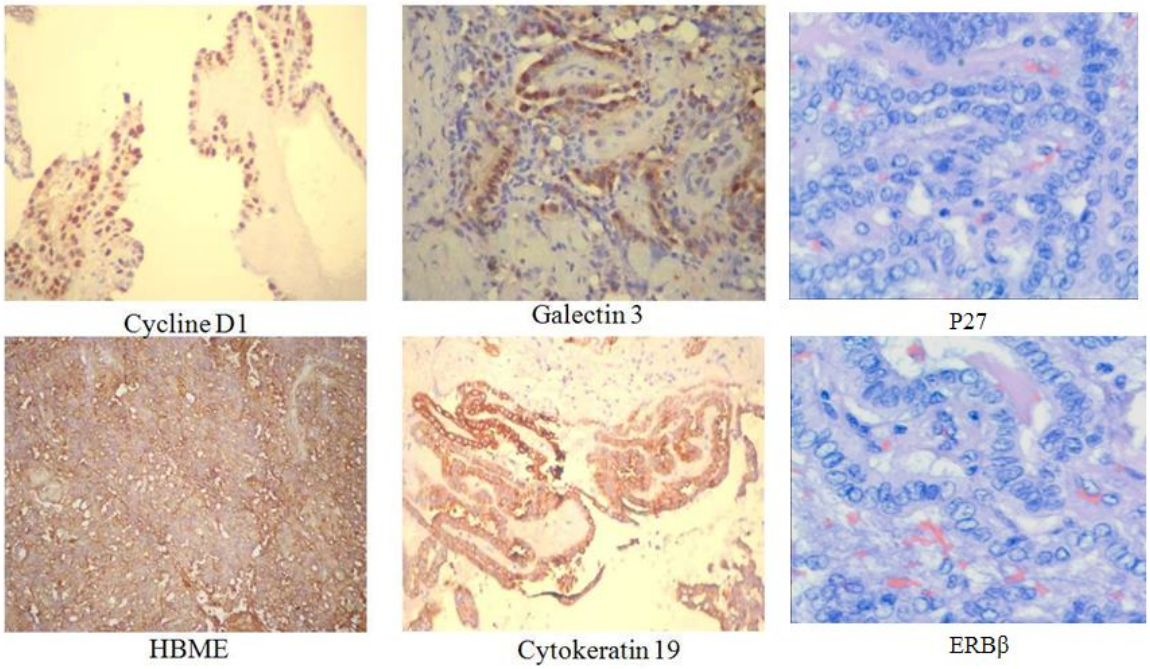


Fig 2.2.6: Immunohistochemical detection of different tumour markers (200X): (A) Cyclin D1: Nuclear positivity in the tumour cells lining the papillae. (B) Galectin3: Nuclear and cytoplasmic positivity observed in tall cells (C) HBME1: Compactly arranged tumour cells show diffuse cytoplasmic and membrane positivity. (D) Cytokeratin19 shows a diffuse cytoplasmic positivity in tumour cells.

2.2.3 Pathological Finding in patient with TCV

The clinical information was retrieved from the patient's medical records. The histological diagnosis was made by two experienced pathologists with hematoxylin and eosin staining.

Completion thyroidectomy specimen showed a solid tumour in perithyroidal soft tissue. It showed papillary and festoon like pattern. At places, papillae were closely packed. The papillae were covered by single layer of tall columnar cells. The tall cell pattern was seen in 90 % of the tumour (Fig 2.2.5). Follicular pattern was totally absent. The height of the tumour cell was two to three times of the width. The characteristic nuclear features of papillary thyroid carcinoma were noted with occasional mitotic activity. The cytoplasm of tumour cells was dense eosinophilic and moderate in amount. The aggressive features like brisk mitotic activity, necrosis and nuclear pleomorphism were seen. Extensive hyalinization was noted. Spindle cell metaplasia was not seen. Psammomatous calcification was absent. Lymphovascular invasion of thyroid epithelial clump of cells were observed (Fig 2.2.6).

In the surgical specimen of recurrence in 2010, the right paratracheal nodes and left paracarotid nodes showed metastasis of papillary thyroid carcinoma with tall cell morphology. Cystic change was observed in the metastatic node. Extranodal extension was also noted. Immunohistochemical markers viz Cyclin D1, Galectin 3, HBME 1, Cytokeratin 19 were unregulated in the recurrent metastatic specimen of the patient. Whereas, P27 and ERBβ protein levels were insignificant in the tumor specimen (Fig 2.2.6).

2.2.4 Genetic analysis

To study the genetic alteration, genomic DNA extraction and PCR amplification of BRAF exon 15 was performed as described in Section 2.1 and as described elsewhere *(189, 240, 244)*. PCR products were sequenced in both forward and reverse directions. PCR reactions in 25µl of final volume were performed as per manufacturer's instructions. Sequences were performed by dideoxy sequencing method using Big-Die Terminator Vre.3.1 kit in ABI Prism 377-18 gel running Sequencer; U.S.A. Sequences were compared by the BLAST program: www.ncbi.nlm.nih.gov/BLAST revealing *BRAF* ^{V600E} and *BRAF* ^{I582M} mutations in exon 15 of *BRAF* gene.

Total RNA from tissue was isolated using method described in Chapter 3.1. cDNA isolated from tumour and adjacent normal tissue of the patient were analyzed with MUC1, BCL2and BCL XL specific primers in Quantitative Real time PCR (Stratagene, USA) using SYBR Green master Mix (CAT # 600548, Stratagene, La Jolla, Ca, USA). The expression of MUC1 gene at the transcriptional level in both normal and cancerous tissue was normalized with their respective β-Actin by $2^{\Delta\Delta ct}$ method *(245, 246)*. Neoplastic tissue showed two fold increases in expression of MUC1 gene in comparison to the normal tissue. Although, the transcript levels of anti-apoptotic gene BCL2 and BCL XL and post translational modification of P53 were similar in tumor tissue compared to normal. (Fig. 2.2.2)

2.2.5 Discussion

PTC accounts for 80-90% of thyroid cancer making it the most frequent follicular cell derived malignancy *(247)*. This is the eighth most common malignancy in women (constituting 3% of all cancers in women) *(189)*. Among the different histotypes of PTC, the tall cell variant is reportedly more aggressive and associated with a less favorable outcome. It shows a high propensity to metastasize in regional lymph nodes as is consistent with our patient and also as reported by others *(229, 232)*. In one report, Adeniran et.al correlated the aggressive behavior of TCV with constitutive activation of MAPK pathway protein B-type Raf kinase *(BRAF) (241, 244)*. The T1799A (exon 15) transversion mutation of *BRAF*, initially reported in thyroid carcinoma by Davies H et al *(209)*, has been described to occur in 30-60 % of thyroid malignancy. It has been shown that *BRAF* plays a critical role as an intermediate in the cAMP induced activation of MEK1 and the extracellular signal regulated kinases in thyrocytes. It has been hypothesized that presence of this oncogenic mutation in PTC would be associated with aggressive disease and unfavorable clinical outcome *(239,248)*. *BRAF* mutation is more prevalent in TCV and possibly responsible for frequent ETE and nodal metastasis. Among 33 mutations reported to occur in BRAF gene, some

mutations like T1746C (I582M) as found in this patient are very rare (Fig 2.2.2). According to Dixit et.al, energy landscape analysis has revealed that mutation has a neutral role in kinase activity and allosteric regulation of BRAF protein *(249)*. In this patient, all the metastatic tissue specimens collected at different times as the disease progressed, showed BRAF V600E mutation (Fig. 2.2.2). According to literature, the presence of BRAF mutation not only increases the risk of lymph node metastasis or difficulty of treatment but also regional nodal relapse. This clearly indicates that persistent presence of constitutively expressing BRAF may have a role in multiple recurrences *(248)*.

Our study also revealed that multiple tumour aggressiveness determination markers were overexpressed in this case. Mucin 1 or polymorphic epithelial mucin (PEM), which is a mucin encoded by the *MUC1* gene in humans, is up regulated at the transcriptional level in the said patient's tumour sample (Fig 2.2.2 C, D). As reported in 2005 by Kapel N et.al over expression of MUC1 glycoprotein "is a key molecular event in pathogenesis of aggressive TC". The heavy glycosylation in the MUC1 prevents the entry of therapeutic agents, access to anti-tumour immune response and augments the accumulation of growth factors *(246)*.

Immunohistochemical analysis of the metastatic tissue sample showed the diffused expression of all the thyroid malignancy specific protein markers (CK19, Cyclin D1, Galectin 3, HBME1) (Fig 2.2.6). Cyclin D1, a cell cycle regulatory protein necessary for Cyclin-CDK complex formation, is reported to be elevated in 60% PTC. Deregulated expression of Cyclin D1 is a key player in molecular mechanism of thyroid carcinogenesis *(250,251)*. The lectin family tumour marker Galectin-3 which is also overexpressed in this patient has a broad biological functionality including cell activation, chemo attraction and carcinogenesis *(252, 253)*. HBME-1 is a valuable marker of follicular variant of thyroid carcinoma and was also found to be overexpressed in this patient. Keratin family tumour marker Cytokeratin 19 which was overexpressed in the tumour tissue is responsible for the structural integrity and

proliferation of epithelial cells *(253, 254)*. All these cell adhesion and cell cycle regulatory proteins are reportedly helpful for differential diagnosis of endocrine malignancy. A combined analysis of these markers that indicate diffuse expression pattern and intense cellular positivity may provide a better prediction for poor prognosis of thyroid cancer. In the clinical practice of thyroid oncology, FDG-PET is now considered an important modality in investigating patients with high Tg and no abnormal iodine concentration in whole body radioiodine scan**.** The valuable diagnostic role of FDG-PET has been emphasized in the literature including revealing second primary in this group of patients *(255, 256)*. In addition to the detection of site of recurrence that can account for raised Tg level, there is now increasing evidence that FDG-PET can predict tumour biology. FDG avid disease is also associated with tumour aggressiveness and relative resistance to radioiodine therapy even in patients of differentiated thyroid carcinoma who demonstrate radioiodine concentration *(257, 258)*. It is logical to hypothesize that the aggressiveness of the disease in terms of clinical behavior could be related with the genetic mutation that is associated with aggressive features in DTC. The present finding in this challenging case also supports this. We thus propose a relation between the BRAF genetic mutation in these lesions and their *in vivo* imaging and histological features as observed in the present case study *(255-259)*. We believe the findings observed in our patient require to be examined in future patients with a similar history and, if proven would open up further research with BRAF specific therapies (e.g. BAY 43-9006, AMG 706, Vemurafenib) in this group of patients.

2.3 Sporadic Point mutations of *RAS* proto-oncogenes

2.3.1 Introduction

2.3.1.1 *RAS* Isoforms:

A family of genes that is frequently found to harbour a mutation in human tumours is that of the *RAS* genes *(260, 261)*. This family consists of three functional genes, *HRAS*, *KRAS*, and *NRAS*, which encode highly similar proteins with molecular weights of 21,000 (Fig.2.3.1) *(262)*. Mutated *RAS* genes were first identified by their ability to transform NIH/3T3 cells after DNA transfection. Subsequent analysis of a variety of tumour samples revealed that one of the three *RAS* genes harboured a point mutation in part of the human tumours; as a result, the protein product has an altered amino acid at one of the critical positions 12, 13, and 61 *(263, 264)*.

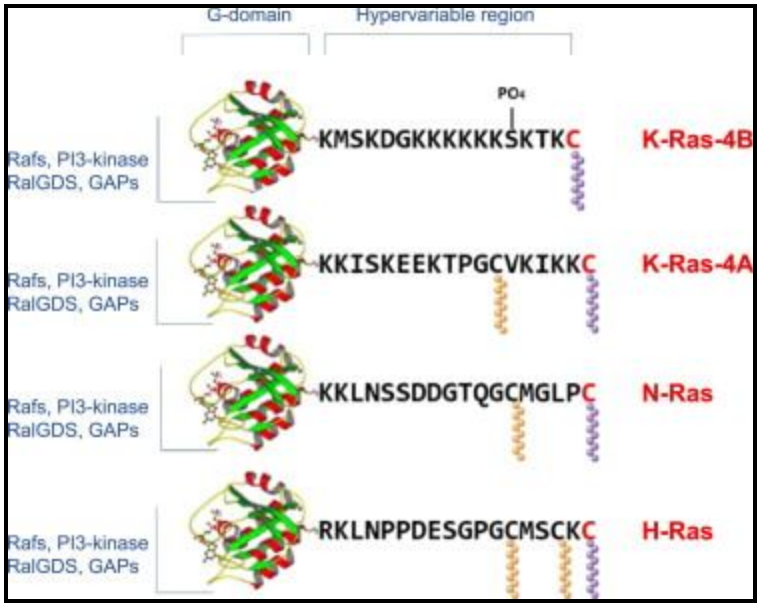


Fig 2.3.1: Schematic Representation of the *RAS* Isoforms The structures of the G domain of *HRAS*, *NRAS*, and *KRAS* have been solved and are virtually identical, but the structure of processed hypervariable regions have not been solved and are, therefore, depicted as a linear sequence. Lipid modifications with farnesyl (purple) and palmitoyl (orange) chains are shown. (Source: Cancer Cell; Volume 25, Issue 3, p272–281, 17 March 2014)

The functional and structural resemblance of the *RAS* proteins with the G-proteins controlling adenylate cyclase has led to the proposal that normal p21*RAS*2 proteins are involved in the transduction of external stimuli, most likely induced by growth factors or factors involved in cell differentiation *(265, 266)*. The current model tells that the *RAS* proteins become activated upon stimulation, transduce the signal to some effector molecule, and subsequently become inactivated (Fig2.3.2). Mutated *RAS* proteins, however, have lost the ability to become inactivated and thus stimulate growth or differentiation autonomously. Despite extensive research, the signals that induce activation of *RAS* proteins and the proteins that are affected by *RAS* proteins in the signal transduction cascade are still unknown. The *RAS* proteins might not be directly linked to cell surface growth factor receptors, but might play a more pivotal role in the transduction of several growth or differentiation factor stimuli *(267, 268)* (Fig 2.3.3).

A protein has been discovered that is involved in the hydrolysis of the GTP bound to *RAS* (Fig 2.3.2). This protein, GTPase activating protein, binds to the effector domain of the *RAS* proteins and might play a role in the transduction of signals from *RAS* to further downstream. Alternatively, other still to be discovered, effector proteins might compete with GTPase activating protein for the effector site *(268, 269)*. The introduction of new and rapid assay systems for the identification of mutated *RAS* genes has made it possible to analyze large numbers of tumour samples for the presence of *RAS* genes. Recently extensive reviews have appeared about the function of *RAS* proteins in both lower and higher eukaryotes, about the role chemical mutagens can play in the induction of the mutation *(270, 271)*, and about the presence of mutant *RAS* genes in human tumour cell lines *(272)*.

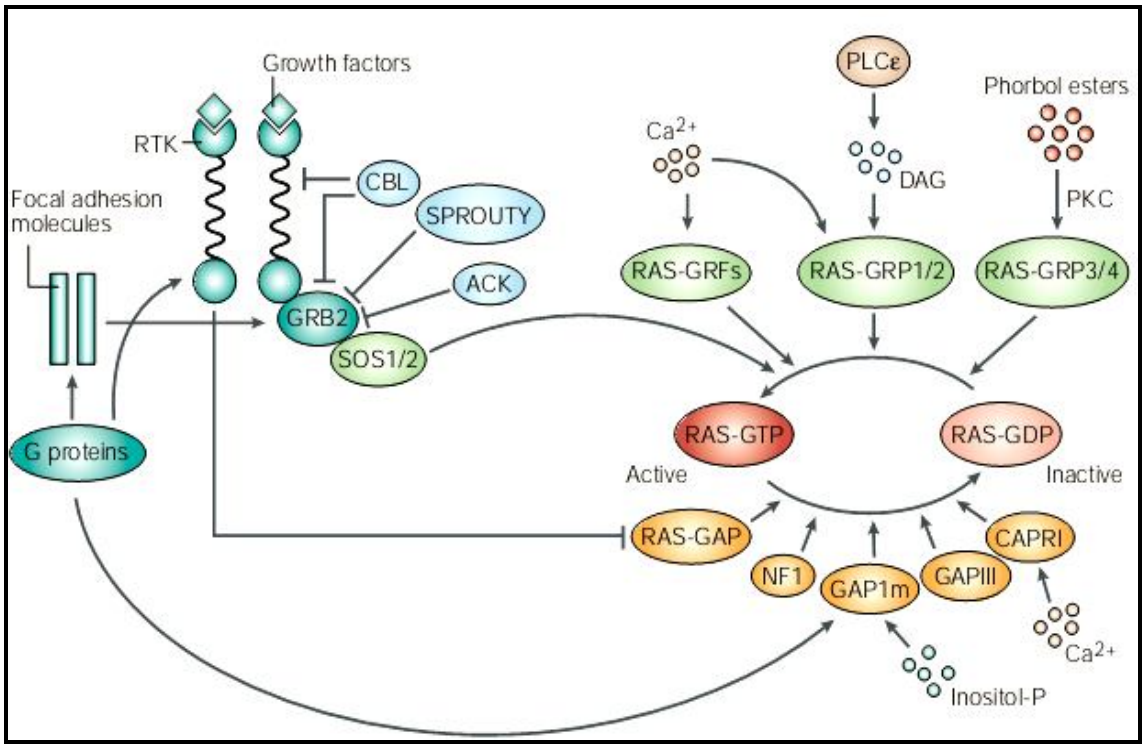


Fig 2.3.2: Schematic representation of our current view of *RAS* regulation. *RAS* proteins are activated by various extracellular stimuli, which are mediated by, among others, receptor-tyrosine kinases (RTKs), G proteins, adhesion molecules and second messengers. These stimuli activate various guanine nucleotide-exchange factors (GEFs; green ovals). Several GTPase-activating proteins (GAPs; orange ovals) are also involved in the downregulation of *RAS* by catalysing the GTP hydrolysis. DAG, diacylglycerol; PLC, phospholipase C. (Source: Nature Review, Cancer, Volume 3, June 2003)

2.3.1.2 Mutations of *RAS* genes in different cancers

The frequency of *RAS* mutations varies in the different sites of human tumours including: pancreas, colon, rectum, small intestine, liver, skin, female reproductive tract, breast, kidney, brain, testis, leukemia and thyroid *(261, 273-275)*.

2.3.1.3 *RAS* in thyroid:

Mutations in all three *RAS* family genes have been found in thyroid tumours. The highest incidence of mutations was found in follicular and undifferentiated carcinomas *(276, 277)* while in papillary carcinomas the incidence of *RAS* mutations was limited *(278)*. Follicular tumours have been known to harbour activating point mutations of the *RAS* genes. Three *RAS* genes, *HRAS*, *KRAS*, and *N-RAS*, encode highly related 21-kDa proteins located at the

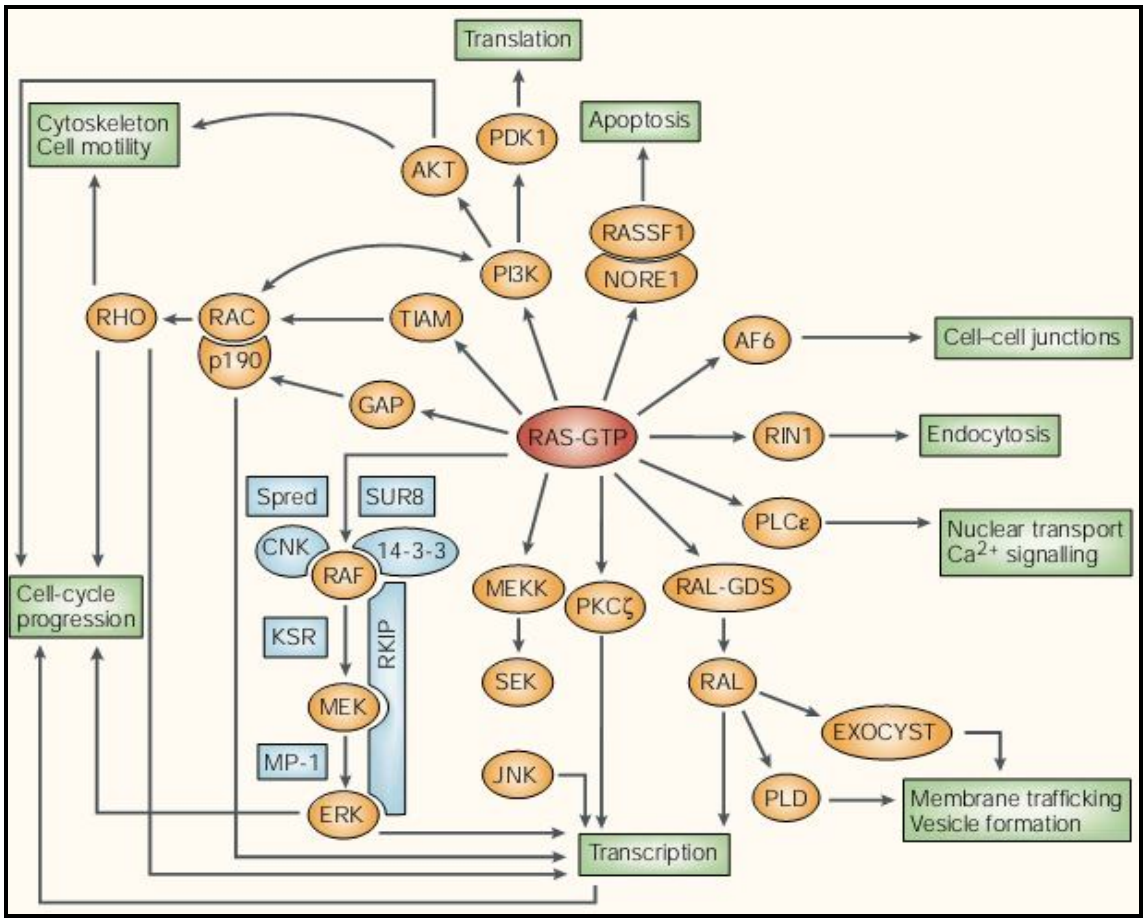


Fig 2.3.3: Overview of known *RAS* effectors and their corresponding biological responses. Active *RAS*-GTP induces a wide variety of cellular processes, such as transcription, translation, cell-cycle progression, apoptosis or cell survival, through direct interaction with various effectors. GAP proteins also interact with *RAS*-GTP and might also act as effectors.(Source: Nature Review, Cancer, Volume 3, June 2003)

inner surface of the cell membrane and play a central role in the transduction of signals arising from tyrosine kinase and G protein-coupled receptors. *RAS* point mutations in thyroid and other human neoplasms occur typically in codons 12, 13, or 61, leading to constitutive activation of downstream signaling pathways in thyroid follicular cells. Somatic missense mutations in codons 12/13 and 61 of one of the three *RAS* genes have been found in 18 –52% of follicular carcinomas *(276, 277, 279-281)* and 24 –53% of follicular adenomas *(277, 279-281)*. A much lower incidence has been reported in Hurthle cell tumours (15–25% of carcinomas and 0 – 4% of adenomas) *(280, 282)*. Macrofollicular hyperplasias are characterized by the absence of mutations in the members of the *RAS* family *(283)*.

2.3.2 Aim and Objective:

Detection of activating point mutations is frequently associated with an aggressive type of the disease and with specific clinical characteristics. The clarification of the role of activated *RAS* alleles in thyroid tumours, may have significant implication in the clinical practice. The presence of mutant *RAS* alleles may serve as molecular markers for the development of the disease *(176, 284)*. Biopsy specimens from surgically resected tumours may be assayed for the presence of *RAS* mutations and this may help to predict the course of the disease or to establish treatment strategies. Furthermore, the detection of *RAS* mutations may provide useful information as regards the prognosis of the disease. Cytological material from FNAC might be used in order to screen the population for the presence of mutant *N/K/HRAS* alleles *(285, 286)*.

A more challenging possibility is the use of the fore mentioned information for the therapy of cancer. Such an approach has been successfully carried out *in vitro* by specific compounds (antisense oligonucleotides) that block *RAS* genes at the level of transcription or posttranscriptional modifications respectively *(287-289)*.

In this study, efforts have been made to investigate 92 patient sample specimens and 3 cell lines. Association of H,N and *KRAS* mutations with detailed clinical parameters have been analyzed, in order to reveal the precise role of the *RAS* family genes in human thyroid cancer and to apply this information in clinical practice in Indian population.

2.3.3 Material and Method:

Tumour Tissue Specimens:

Frozen and paraffin embedded thyroid tissues from thyroid cancer patients who had attended the clinic between 2000 and 2010 were retrieved from the Tissue Repository of Tata Memorial Hospital, Mumbai, India for analysis of *RAS* gene mutations at codon 12, 13 and 61. A total of 92 specimens with adequate clinical and pathological information were studied. Tissue specimens included 14 PTC, 14 FTC, 3 FTA, 25 FVPTC and 33 PDTC.

Patients were staged using the tumour-node-metastases (TNM) system and classified according to the presence of extra thyroidal extension, cervical nodes and distant metastases.

Cell Lines:

Three thyroid tumour cell lines were used in this study including follicular carcinoma (WRO) and anaplastic carcinoma (ARO and FRO). The cell lines have been discussed in details in the first part of this chapter.

Molecular Analysis:

Genomic DNA Extraction:

Nucleic acid extraction was performed as described previous in Chapter 2, Section 2.1.

SNP analysis using KASP technology:

The Competitive Allele Specific PCR (KASP) genotyping assay was utilized for *RAS* gene SNP analysis of DNA samples. KASP is a unique form of competitive allele-specific PCR combined with a novel, homogeneous, fluorescence-based reporting system for the identification and measurement of genetic variation occurring at the nucleotide level to detect

single nucleotide polymorphisms (SNPs) or inserts and deletions. The KASP technology is suitable for use on a variety of equipment platforms and provides flexibility in terms of the number of SNPs and the number of samples able to be analyzed. The KASP chemistry functions equally well in 96, 384, and 1,536 well micro-titer plate formats and has been utilized since many years in large and small laboratories by users across the fields of human, animal, and plant genetics *(290-292)* (Fig. 2.3.4).

The KASP method is a cost effective method over multiplex methods. There is also a much shorter turnaround time to receive the results with the KASP method than other multiplex methods. Additionally, there is a lower genotyping error rate of 0.7-1.6%.

The analysis was performed using three initial steps 1) designing of two allele-specific forward primers, 2) dilution of purified DNA sample, and 3) designing of a common reverse primer. 50 bp upstream and downstream of SNP sites were selected using online Basic Local Alignment search tool (BLAST) in National Center for Biotechnology Information (NCBI) (Table 2.3.1). A 60 µl volume of 15 ng/µl of the extracted DNA sample were aliquoted in each of the 96 well PCR plate and sealed. The sealed plates with DNA were sent for analysis to LGC-Genomics, Germany, where the forward and reverse primer sets were designed. The probe containing the fluorophore and quencher were also designed.

Mutations were detected with bi-allelic scoring using the following steps. In the first round of PCR, the KASP primer mix that contains the two allele-specific forward primers and the single reverse primer is added to the mixture.

SNP ID	Sequence	Chromosome	C position	Genetic alteration	Amino Acid Change
NRAS C12GC	ccaacaggttcttgctggtgtgaaatgactgagtacaaactggtggtggttggagca[g/t]gtggtgttgggaaaagcgcactgacaatccagctaatccagaaccactttgt	Chromosome1 p 13.2	12	GGT >TGT	Gly>Cys
NRAS C13GC	ccaacaggttcttgctggtgtgaaatgactgagtacaaactggtggtggttggagcaggt [g/t]gtgttggga aaagcgcact gacaatccag ctaatccaga accactttgt	Chromosome1 p 13.2	13	GGT >TGT	Gly>Cys
NRAS C61QL	acaagtggttatagatggtgaaacctgtttgttggacatactggatacagctggac[a/t]agaagagtacagtgccatgagagaccaatacatgaggacaggcgaaggcttcc	Chromosome1 p 13.2	61	CAA >CTA	Gln>Leu
KRAS C12GD	ataaggcctgctgaaaatgactgaatataaacttgtggtagttggagctg[g/a]tggcgtaggcaagagtgccttgacgatacagctaattcagaatcattttgtggacgaat	chromosome 12 p12.1	12	GGT >GAT	Gly>Asp
KRAS C12GS	ataaggcctgctgaaaatgactgaatataaacttgtggtagttggagct[g/a]gtggcgtaggcaagagtgccttgacgatacagctaattcagaatcattttgtggacgaat	chromosome 12 p12.1	12	GGT >AGT	Gly>Ser
KRAS C13GD	ataaggcctgctgaaaatgactgaatataaacttgtggtagttggagctggtg[g/a]cgtaggcaagagtgccttgacgatacagctaattcagaatcattttgtggacgaat	chromosome 12 p12.1	13	GGC >GAC	Gly>Asp
HRAS C13GD	gcaggcccctgaggagcgatgacggaatataagctggtggtggtgggcgccggcg[g/a]tgtgggcaagagtgcgctgaccatccagctgatccagaaccattttgtggacga	chromosome 11 p15.5	13	GGT >GAT	Gly>Asp
HRAS C61QL	gcaggtggtcattgatggggagacgtgcctgttggacatcctggataccgccggcc[a/t]ggaggagtacagcgccatgcgggaccagtacatgcgcaccgggggagggcttcc	chromosome 11 p15.5	61	CAG >CTG	Gln>Leu
BRAF C15VE	atatatttc ttcatgaaga cctcacagta aaaataggtg attttggtct agctacag[t/a]g aaatctcgat ggagtgggtc ccatcagttt gaacagttgt ctggatccat	chromosome 7 q 34	15	GTG >GAG	Val>Glu

Table 2.3.1: Chromosomal location of SNP of H, N and *KRAS* genes with upstream and downstream sequences

The specific nature of the forward primers allows for the primer to bind solely at the SNP of interest, allowing DNA polymerase to lay down the rest of the complementary nucleotides. During this time, the common reverse primer begins to lay down complementary nucleotides on the opposite strand of DNA. This ends the first round of PCR.

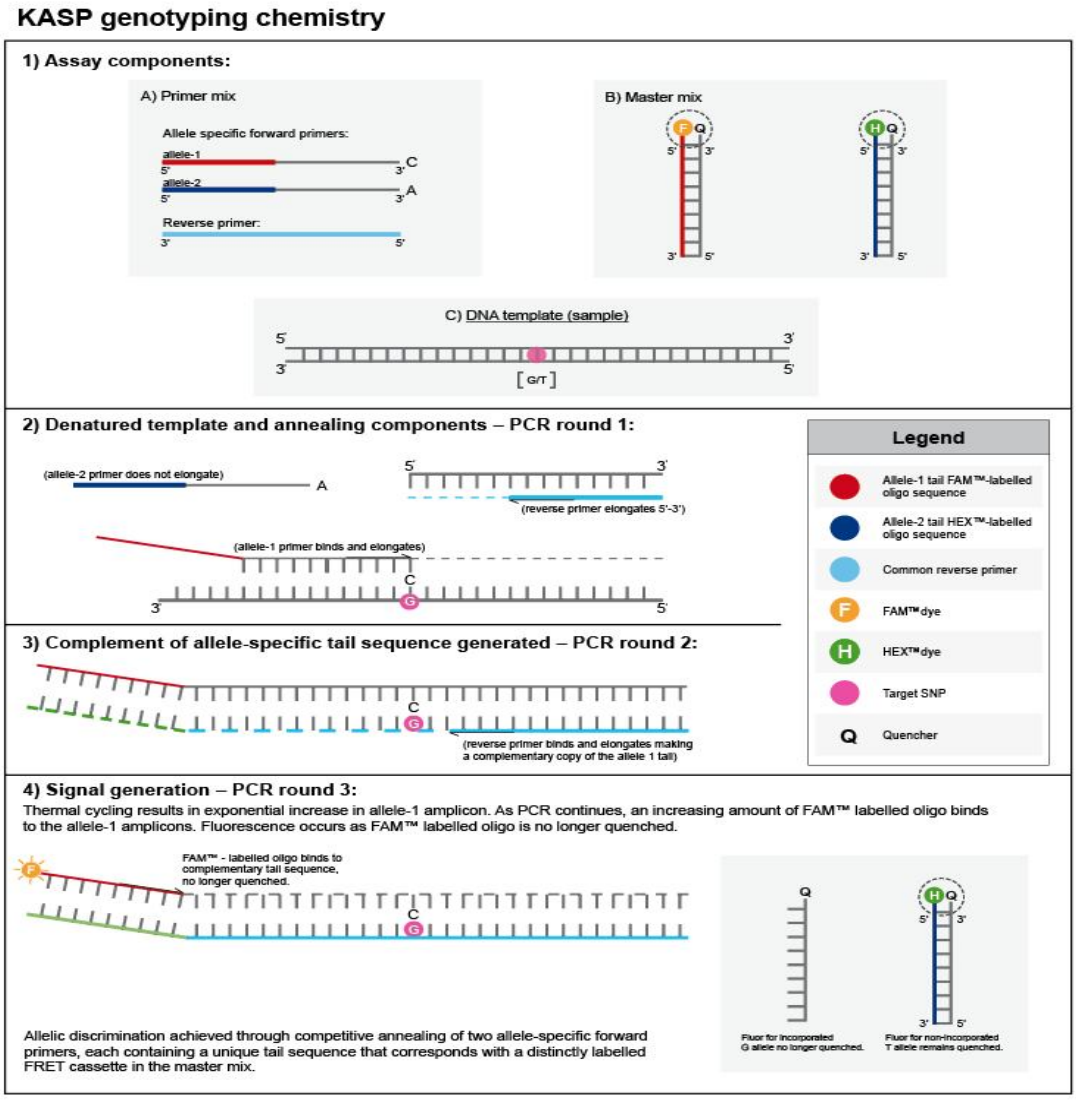


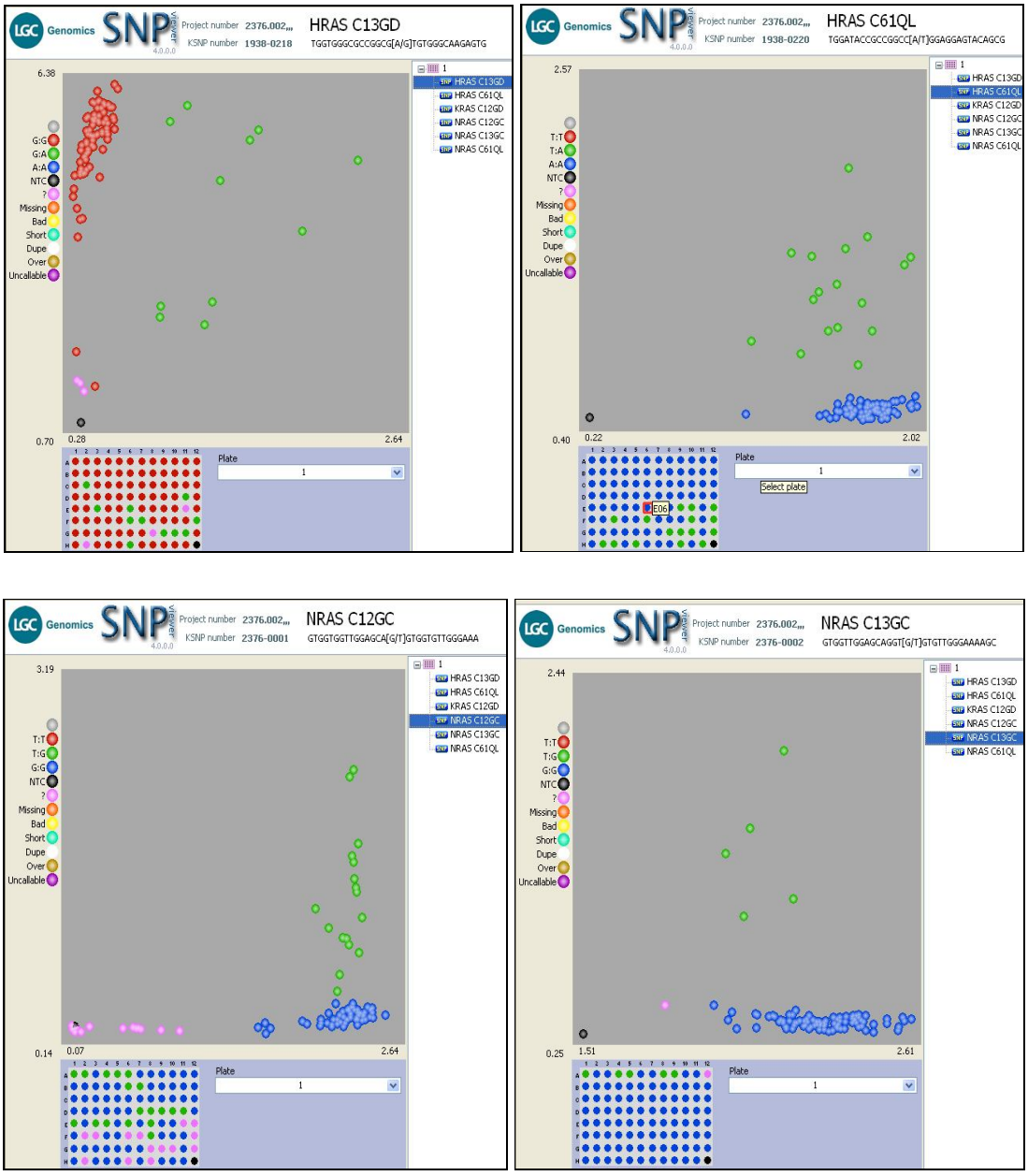
Fig2.3.4: Schematic drawing of KASP chemistry. (Source: http://www.kbioscience.co.uk)

In the second round of PCR, a compliment to the allele-specific forward primer is generated when the common reverse primer binds to the amplicon formed in the first round of PCR. Finally, the thermocycling of the PCR reaction continues, starting the third portion of the KASP method. The FAM oligo, a fluorescently labeled primer found in the master mix, complements the tail sequence of the common reverse primer, allowing for elongation to occur. This occurs multiple times throughout the thermocycling settings and the fluorescent signalling becomes stronger as more FAM oligo primers are used in the amplification process.

2.3.3 Results:

2.3.3.1 *RAS* Mutation Pattern:

The KASP analysis of common types of *RAS* mutations are illustrated in Figure 2.3.5. The X and Y coordinates represent the end point fluorescence intensity of the fluorophore labeled probe that are bound to the wild type and mutant allele specific PCR amplicons.



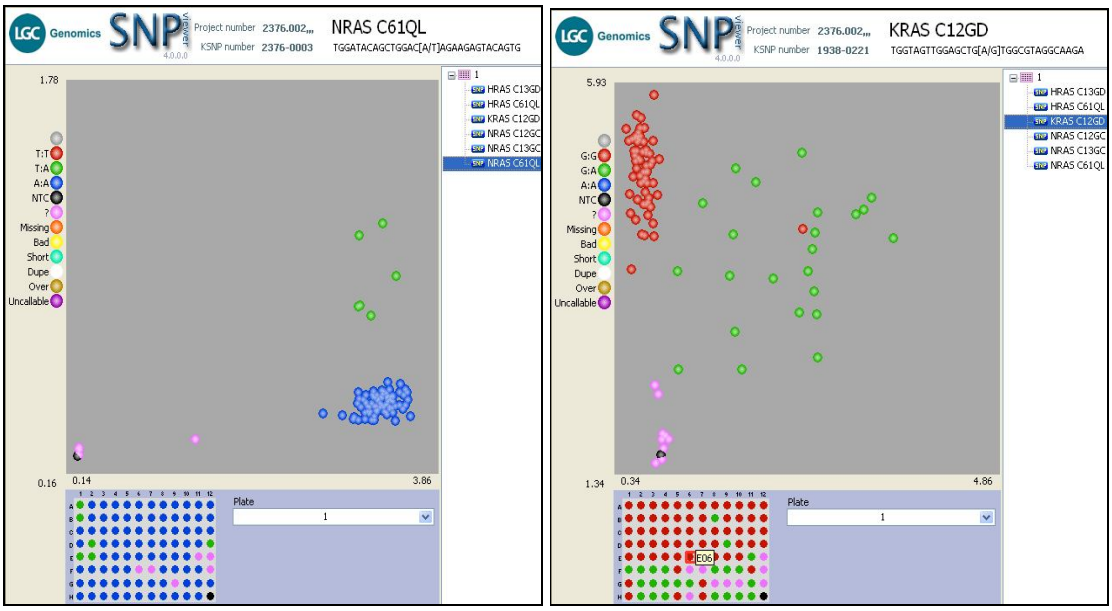


Fig 2.3.5: Snapshot displaying wild type and mutant alleles by genotyping with Kompetitive Allele Specific PCR (KASP) assay.

The results of H, K and *NRAS* mutation analysis and the correlation of *RAS* mutation status with clinico-pathologic features are summarized in Tables 2.3.2.

Among the mutations identified in different *RAS* isoforms, the *KRAS* C12GD mutation is of highest prevalence (23 of 95 samples, 24.2%), all of them occurring at codon 12. *NRAS* C12GC mutation was found in seventeen (17.8%), *NRAS* C13GC mutation in five (5%) and *NRAS* C61QL mutation in six (6.3%) out of 95 neoplasias. Mutation in *HRAS* gene at codon 13 and 61 were found in eleven (11.5%) and sixteen (16.8%) tumours respectively. Both transition and transversion type of nucleotide changes were equally apparent.

	HRAS C13GD	*HRAS* C61QL	*NRAS* C12GC	*NRAS* C13GC	*NRAS* C61QL	*KRAS* C12GD
No of Cases	11	16	17	5	6	23

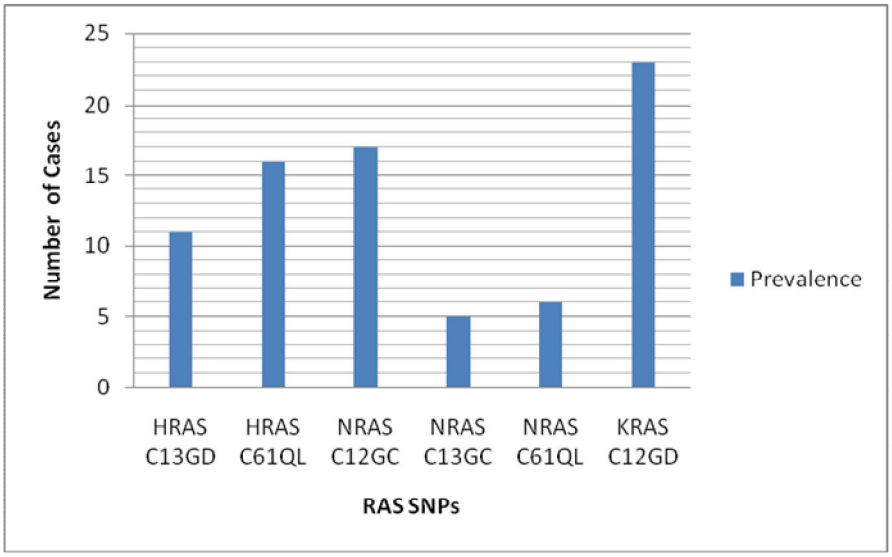


Fig2.3.6: Number of cases with different *RAS* mutations

Among the different histological subtypes, *RAS* mutation was found in 57 out of 95 samples (60%) of Indian cohort. Percent incidence of this mutation varied among pathological subtype. *RAS* gene mutation was much more common in the PDTC (29 out of 35; 82.8%) than in the PTC (7 out of 14; 50%), FTC (19 out of 42; 45.2%). Two out of 4 ATC (50%) were harbouring *RAS* mutation. Multiple activating mutations were restricted to PDTC (28%) and ATCs (50%). 10 of the 35 PDTC and 2 out of 4 ATCs harboured multiple *RAS* mutations. (Table 2.3.2)

Table 2.3.2: Prevalence of RAS gene mutation

Histology	Total (N)	*RAS* Mutations	
		Positive (n1)	%
Total	95	57	60
Papillary Carcinoma	14	7	50
Follicular Carcinoma	42	19	45.2
Poorly Differentiated	35	29	82.8
Anaplastic Carcinoma	4	2	50

Table 2.3.3: Prevalence of *HRAS* and *KRAS* Mutations in 94 thyroid neoplasia and cell lines

Histology	Total (N)	*HRAS* C13GD		*HRAS* C61QL		*KRAS* C12GD	
		Positive (n1)	%	Positive (n2)	%	Positive (n3)	%
Papillary Carcinoma	14	0	0	0	0	0	0
Follicular Carcinoma	42	4	9.7	2	4.8	3	7.3
Poorly Differentiated	35	7	20	12	34.2	17	48.5
Anaplastic Carcinoma	4	0	0	2	50	2	50

HRAS (C13GD and C61QL) is observed in 7 (20%) out of 35 PDTCs followed by 4 (9.7%) out of 41 patients of FTCs. PDTC also harboured *KRAS* C12GD mutation in 17 (48.5%) cases. However, none of the 14 PTCs contained *HRAS* (C13GD and C61QL) or *KRAS* C12GD mutation. (Table 2.3.3 and 2.3.4)

NRAS mutation at codon 12, 13 and 61 were found in higher frequency in PTC (28%, 28% and 14% respectively) with respect to other histotypes. Mutation at codon 13 was exclusively

present in PTC and PTC derived PDTC (2.8%) and not in FTC. Also, none of the ATCs showed *NRAS* mutation.

Table 2.3.4: Prevalence of *NRAS* Mutations in 94 thyroid neoplasia and cell lines

Histology	Total	*NRAS* C12GC		*NRAS* C13GC		*NRAS* C61QL	
		Positive	%	Positive	%	Positive	%
Papillary Carcinoma	14	4	28.5	4	28.5	2	14.2
Follicular Carcinoma	42	11	26.8	0	0	4	9.7
Poorly Differen.	35	2	5.7	1	2.8	0	0
Anaplastic Carcinoma	4	0	0	0	0	0	0

2.3.3.2 Correlation of *RAS* with Clinico-pathologic Parameters:

The association between *RAS* mutations and various clinico-pathological parameters were assessed in 94 samples (Table 2.3.5) by Fisher's exact test, univariate and multitvariate logistic regression analysis. By Fisher's test *NRAS* mutation status showed association with histotypes of thyroid carcinoma ($P<0.05$) and lymph node metastasis ($P<0.05$). But no correlation was found with age, gender and TNM Stage, tumour volume and extra-thyroidal extension. Multivariate analysis performed with the same clinico-pathological parameters revealed correlation of *NRAS* with only histological type (odds ratio = 0.69; 95% confidence interval, 0.48-0.98, $P<0.05$) (Table 2.3.5).

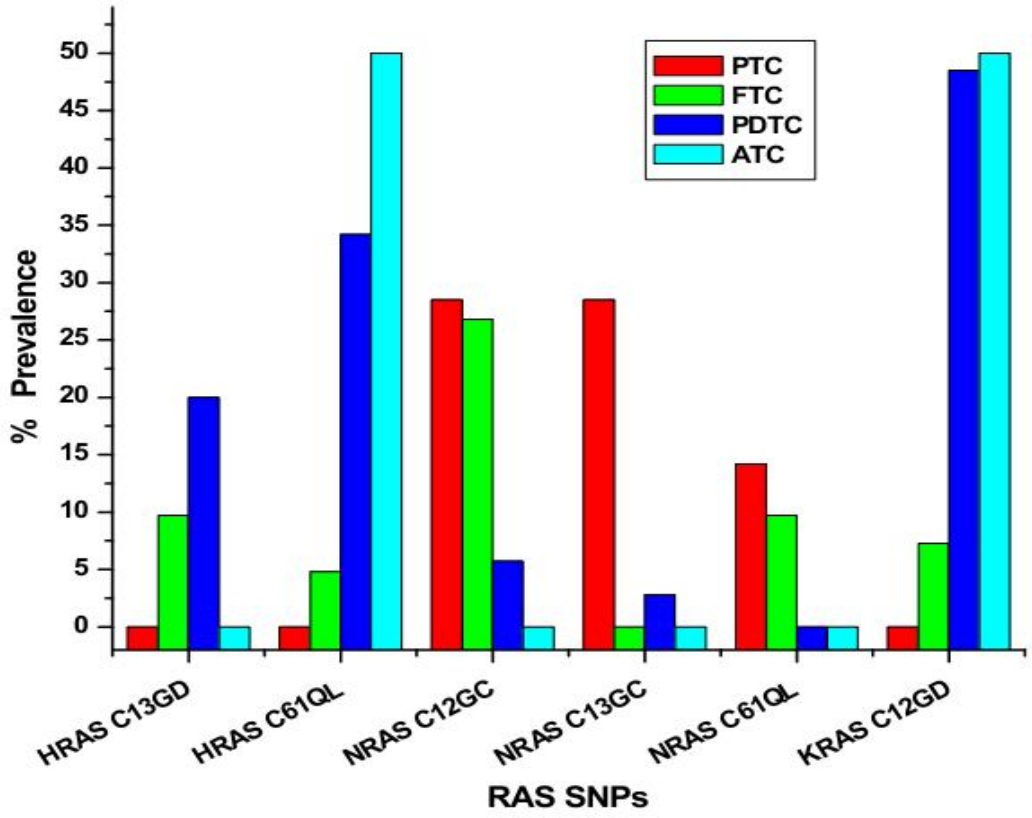


Fig 2.3.7: Comparative representation of prevalence (%) of mutated *RAS* isoforms in different Thyroid Carcinoma Histotypes

There was significant association between *KRAS* and *HRAS* mutation with tumour subtypes ($p<0.005$ and $p<0.001$ respectively) by univariare regression analysis. A multivariate analysis was performed with the same clinico-pathological parameters and were correlated with *KRAS* and *HRAS* mutation (odds ratio = 2.5; 95% confidence interval, 1.36-4.77, $P<0.005$; odds ratio = 3.64; 95% confidence interval, 1.47-9.0, $P<0.006$) with and without stepwise multiple logistic regression method.

All the tumours were divided into morphological subtypes, where FTC was the largest group. Correlation of *BRAF* mutation with the same parameters was sought within this group. However, no significant correlation, other than association with tumour type, was found either by univariate or by multivariate analysis.

Table2.3.4: Correlation between *RAS* mutation and clinico-pathological parameters in papillary thyroid carcinoma

	NRAS				Fisher's test (2 t)	Multivariate Analysis	
	Positive		Negative		*P*	*P*	Odds ratio(95%CI)
	N	%	N	%			
Tumour Stage					0.25	0.12	2.7 (0.75-9.7)
T I	4	13.7	25	86.2			
T II	6	21.4	22	78.5			
T IIIIV	13	30.9	29	69			
Gender					0.622	0.466	1.49(0.5-4.3)
Male	10	28.5	25	71.4			
Female	13	22.8	44	77.2			
Age					0.48	0.428	0.646 (0.219-1.9)
≥45yr	11	22	39	78			
<45yr	12	28.5	30	71.5			
Metastasis					0.049*	0.754	1.2(0.2-7.7)
Yes	18	32.7	37	67.2			
No	5	13.5	32	86.4			
Type	-	-	-	-	-	0.041*	0.691 (0.48-0.98)
Extra-thyroidal Extension					0.344	0.067	0.27 (0.75-9.7)
Yes	11	21.1	41	78.8			
No	12	30	28	70			

*** p<0.05**

2.3.4 Discussion:

Activating *H, K,* and *NRAS* mutations represent the most common type of abnormality of a dominant oncogene in human cancer and have been identified in many different types of tumours, with specificity and type of mutation varying in relation to the tumour type *(260, 293)*. Previous studies have addressed the relationship between *RAS* mutations and the

clinicopathologic features of the tumours harbouring the mutation in different ethnicities. Several studies, including a prospective study *(294)* and large meta-analyses, *(295, 296)* have shown that *RAS* mutations are associated with poor prognosis in colorectal adeno-carcinoma and that different gene mutations have different prognostic impact *(294)*.

Both prospective *(297)* and retrospective *(298, 299)* analyses have shown that *KRAS* mutations are associated with poor prognosis in non–small-cell lung carcinoma. The type of *KRAS* mutation may also influence survival in pancreatic adeno-carcinoma, *(300)* whereas N-*RAS* mutations are associated with failure to achieve complete remission in acute myeloid leukemia *(301, 302)*.

Constitutive activation of all three *RAS* oncogenes (*H, K* and *NRAS*) has recently been reported to occur among tumours that originated from the follicular epithelium of the thyroid gland *(276, 303)*. However, there are significant discrepancies related to the overall frequency of *RAS* mutations (ranging from 7% to 62%) *(303-305)* and their prevalence in specific thyroid tumours. No consistent relationship between tumour histotype or biologic behavior and one particular pattern of *RAS* activation can be inferred from a review of the literatures. Although it is difficult to explain this lack of consistency, the mutation screening methods, the selection of patients, and the design of individual studies may be responsible for the variations in associations between *RAS* mutational status and clinical or pathologic parameters.

We have used Competitive Allele Specific PCR method (KASP), a technique that, with much lower turnaround time to receive the results and minimal genotype error rate (0.7 to 1.6% depending on starting DNA quality) *(292)*. We have also selected tumours that include the full spectrum of different histotypes in follicular cell derived thyroid cancer with a large number of patients. This allowed us to perform a meaningful statistical analysis.

This study demonstrates that different isoforms of *RAS* mutations define a specific subset of thyroid carcinoma. This is indicated by the close association between oncogenic *NRAS* C13GC with PTC and HRS C13GD with PDTC. Exclusive presence of *KRAS* C12GD with PDTC and ATC indicates towards the probable role of this activating mutation in the loss of those histologic features that characterize well differentiated thyroid tumour phenotypes *(53, 303)*. Reportedly, oncogenic *KRAS* not only correlates with the loss of tumour differentiation but also with the presence of distant metastases and response to chemotherapy *(306, 307)*. This is inconsistent with our study as we could not find correlation of *KRAS* C12GD with invasiveness and metastasis *(306)*. Analysis of *KRAS* codon 13 and 61 (results awaited) may have answers towards this discrepancy. Relevantly, we found relative higher prevalence of *RAS* mutations in PDTC compared to FTCs. However, after detailed investigation of the reported PDTCS it was understood that they are prevalently of FTC origin. This further supports the role of *HRAS* and *KRAS* in dedifferentiation of well differentiated follicular thyroid carcinoma.

In summary, our results predict that *RAS* gene mutations have association with different histological subtypes of thyroid carcinoma. Among different *RAS* mutation *NRAS* mutations may be a potential prognostic marker for aggressive behavior and poor outcome of thyroid cancer. Although additional investigations of remaining activating mutations of *KRAS* (codon13, 61) and *HRAS* (codon 12) are necessary to elucidate further the relationship between *RAS* oncogene activation and thyroid neoplasia, our results indicate that *RAS* genotyping may be of significant value as a prognostic indicator and may provide the rationale for novel treatment modalities.

Chapter 3

Chromosomal Rearrangements in Thyroid Cancer

3.1 Introduction

Most of the cancer-causing genes are altered by chromosomal rearrangements, a process that creates a chimeric gene or apposes a gene to the regulatory elements of another gene *(308, 309)*. So far chromosomal rearrangements were most commonly observed in leukemias, lymphomas and mesenchymal tumours, but now have been reported in epithelial tumours involving the thyroid, kidney and breast *(310, 311)*.

There are two types of chromosomal rearrangements. In one type of rearrangement, there is an exchange of genetic material between two different chromosomes that is known as chromosomal translocation (interchromosomal rearrangement). Whereas in the other type, there is an exchange of genetic material between loci on the same chromosome, known as chromosomal inversion (Intrachromosomal rearrangement) *(312, 313)*. One of the major interchromomal rearrangement observed in thyroid cancer is PAX8/PPAR γ translocation, described in Section 3.2. Another most prevalent intrachromosomal rearrangement in thyroid neoplasia is *RET/PTC* rearrangement, elaborated in section 3.3 *(283, 309, 314)*.

3.2 PAX8/PPAR γ Fusion Oncogene in Thyroid Cancer

3.2.1 Introduction to PAX8/PPAR γ

When identified at early stages, most well-differentiated thyroid cancers are readily treated and yield excellent outcomes. In particular, follicular thyroid cancer (FTC) when diagnosed at a late stage, may be very resistant to treatment, and exhibits 10-year survival rates of less than 40% in patients *(315)*. Despite substantial progress in recent years, we still have only a limited understanding of the molecular and biological interrelationships between various subtypes of benign and malignant follicular thyroid neoplasms. In contrast to the wealth of information available regarding papillary thyroid carcinoma (PTC), the triggering mechanisms of FTC development and the major underlying genetic alterations leading to follicular thyroid carcinogenesis remain obscure.

A substantial body of evidence suggest that the PTEN/PI3K/AKT pathway is frequently involved in the pathogenesis of FTC and ATC *(316, 317)*. In addition, recent studies focusing on chromosomal rearrangements in FTC have implicated PPAR mediated pathways *(115)* and are the focus of much research. However, the pathogenesis of FTC remains much less well defined than that of PTC. Since FTC are among the more aggressive tumours and account for a larger proportion of the mortality associated with the disease, continued effort to understand the molecular pathogenesis of FTC is required with the hope of establishing targets for development of alternative therapeutic strategies.

3.2.1.1 Chromosomal rearrangements in follicular thyroid cancer

An important, relatively recent development in the study of FTC tumourigenesis has been the identification of the *PAX8/PPARγ* fusion oncogene. This fusion oncogene is created by a balanced translocation between chromosomes 2 and 3 *(115)*. During the chromosomal exchange, the 2q13-qter region is translocated to 3p25, resulting in an in-frame fusion

between most of the coding sequence of the thyroid-specific paired-box transcription factor PAX8 (2q13) and the entire translated reading-frame of the gene of the liganded nuclear receptor-family member per-oxisome proliferators activated receptor gamma (PPAR) (3p25) (Fig 3.2.1). Several studies have implicated the role of expressed novel PAX8/PPAR fusion protein (PPFP) in the pathogenesis of FTC. The PPFP fusions exhibit several different PAX8 breakpoints, although the PPAR breakpoint appears to be constant *(115, 318)*.

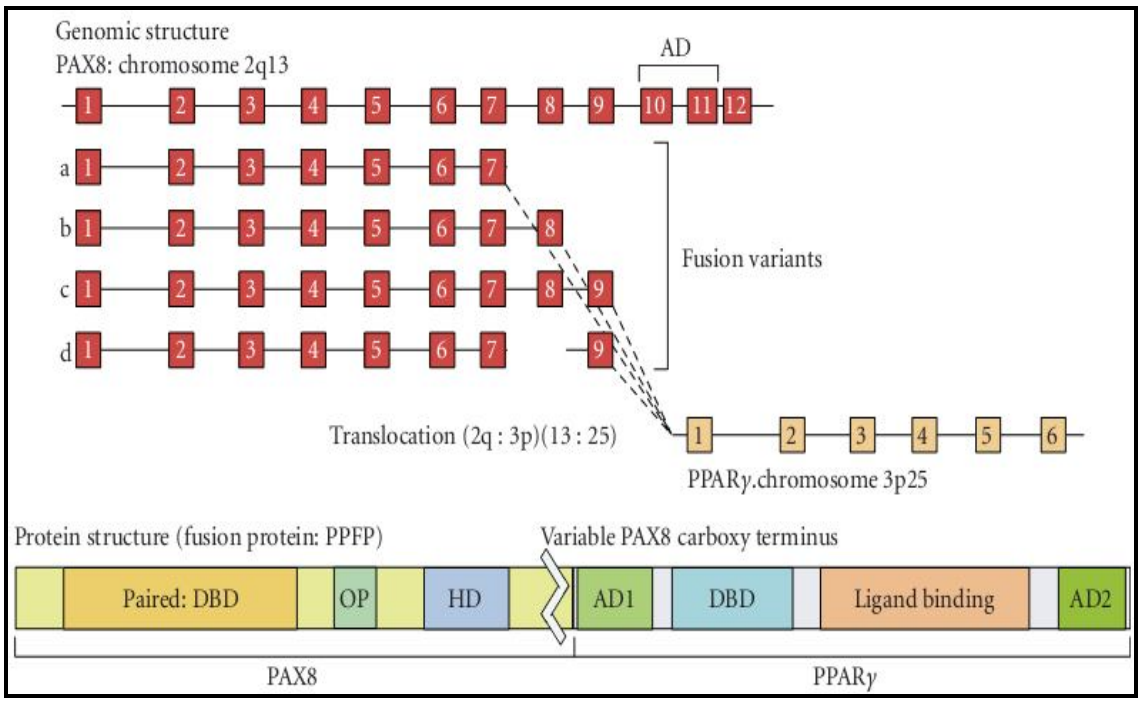


Figure 3.2.1. Pax8/PPARγ rearrangement illustrating the genomic structure with exon arrangement and sites of fusion. The PAX8 activation domain (AD) is eliminated in all fusion events. The protein structure of the predicted fusion protein is shown and contains the PAX8-paired domain, containing the DNA binding domain (DBD), the octapeptide motif (OP), and the truncated homeodomain (HD). All of the functional domains of PPAR γ gene, including activation domains 1 and 2 (AD1 and AD2), DBD, and ligand binding domain are retained in the fusion protein. (Source: PPAR Research, Volume 2008, Article ID 672829,10 pages)

PAX8 plays an essential role in the terminal differentiation steps of thyrocyte development *(319)* and, unlike other members of the PAX gene family, is a key regulator of terminally differentiated gene expression, including the sodium-iodide symporter, *(320, 321)*. Thus

interference with PAX8 function by PPFP could represent a conceivable mechanism by which PPFP influences tumourigenesis. The PAX8 gene promoter drives expression of the PPFP gene *(115)*. Accordingly, PPFP expression would be expected to correlate with the differentiation status of thyroid tumours, being high in well-differentiated tumours and low or absent in poorly differentiated tumours *(322, 323)*. PPFP has been identified in about 36% of FTC, 11% of follicular adenoma (FA), 13% of the follicular variant of PTC (FVPTC), and 2% of Hurthle cell carcinoma and is absent from PTC, anaplastic thyroid cancer (ATC), and benign nodular hyperplasia *(324)*. The occurrence of PPFP in FA, a benign condition, raises questions whether PPFP causes cancer; however, if there is an FA to FTC progression, PPFP may play a role in the progression pathway (Fig. 3.2.2). On the basis of its occurrence, its expected PAX8-dependent expression pattern, and its oncogenic functions, it has been proposed that PPFP may represent an early FTC-specific oncogene *(176, 283, 323)*.

PPFP mediated interference with PPAR function is another potential target for PPFP's oncogenic actions. While PPAR γ is widely recognized to regulate adipogenesis and plays a major role in insulin sensitization, in recent years it has also been studied for its potential role in tumourigenesis *(325, 326)*. In addition, PPAR ligands have increasingly been evaluated as therapeutic agents in a variety of cancers *(327, 328)*, including thyroid cancer *(329)*. The influence of PPAR in the pathogenesis of cancer may be mediated in part by its roles in regulating cell cycle control and apoptosis through its influence on gene expression involving multiple cell signaling pathways *(330,331)*.

3.2.1.2 Role of PAX8/PPAR as an oncogene

Several *in vitro* studies from a number of laboratories have provided evidence that PPFP can act as an oncogene. Powel et al. have utilized both transient and stable transfection of PPFP in Nthy-ori 3-1 cells and have shown accelerated growth rates and lower numbers of cells in the G0 /G1 resting state in PPFP-transfected cells compared to expression vector *(322)*.

Similar results on growth and apoptosis rates have been observed in cells that have been stably transfected with PPFP *(322)*. The over-expression of both PPFP and CREB3L2/PPAR stimulated the proliferation of primary human thyroid cells *(332)*, suggesting that the common PPAR moiety is an essential component of these fusion genes' dominant oncogenic mechanism of action. PPFP transfection also reduced dramatically the rates of apoptosis in the PPFP positive cells, suggesting reduced apoptosis may explain much of the enhanced growth *(322)*. Also, PPFP expression in FRTL-5 cells leads to enhanced proliferation as assessed by ^{3}H-thymidine incorporation and soft agar assays *(333)*. Taken together these studies suggest that PPFP expression may provide a significantly faster as well as promote attachment-independent growth, both factors that contribute to tumourigenesis. While PPFP did not directly affect tumourigenesis, studies provide strong evidence that PPFP acts as a dominant negative inhibitor of wild-type PPAR and affects epithelial cell behavior and carcinogen-induced tumourigenesis, as discussed in more detail below. While studies demonstrate that PPFP exerts oncogenic functions *in vitro*, there is as yet no demonstration of PPFP's ability to affect tumourigenesis *in vivo*. Thus the impact of PPFP on the behavior and biology of human FTC remains to be established. Also, it remains to be seen, whether PPFP acting alone is sufficient to promote tumourigenesis, or whether additional oncogenic events are required for its action. Clearly, additional studies will be required to understand the importance of these other factors and their interrelationships, if any, to PPFP-mediated tumourigenesis.

3.2.1.3 Molecular Mechanism of PPFP Action

While the precise mechanism of PPFP action remains to be elucidated, different studies demonstrate that PPFP can act as a dominant negative inhibitor of wild-type PPAR activity and/or as a unique transcriptional activator of PPAR and PAX8-responsive genes (Fig. 3.2.2).

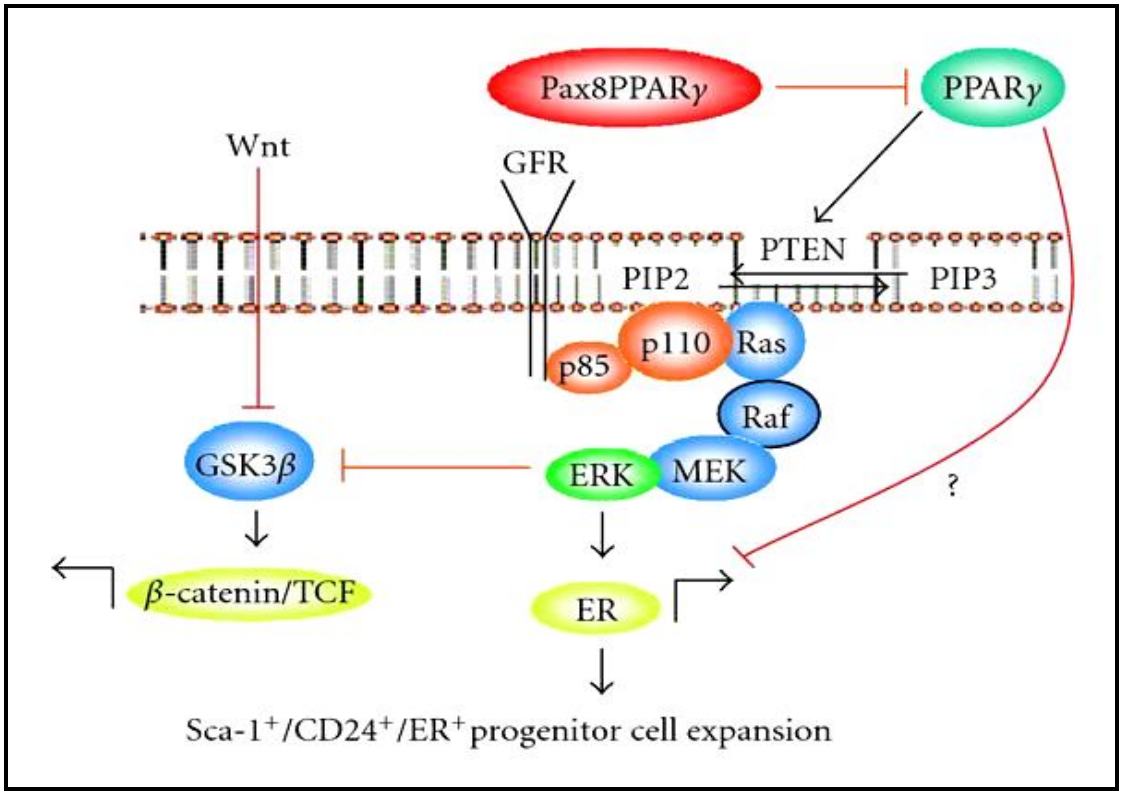


Figure 3.2.2: Figure showing the influence of *PAX8/PPARγ* fusion protein over PTEN: PPARγ (as shown in the figure) activates PTEN, whose major role is to regulate cell proliferation and cell migration. PTEN does this by inhibiting the phosphorylation of Shc, component of MAPK pathway; and FAK phosphorylation, component of integrin mediated pathway. However the fusion protein, *Pax8/PPARγ*, acts in a dominant-negative fashion to block PPARγ-dependent transactivation and upregulation of PTEN. MMTV-*Pax8/PPARγ* mice exhibit reduced PTEN and activation of Ras and ERK, presumably through activation of PI3K (*p85 and p110*). ERK activates ER transcriptionally and posttranslationally, and *Pax8/PPARγ* interferes with the ability of PPARγ to inhibit ER transactivation. (Source: Glazer RI et.al, PPAR Research 2008)

PPFP is also capable of both stimulating and inhibiting selected PAX8-responsive genes *(333)*. In addition, at least under some *in vitro* conditions PPFP can exert independent, ligand-responsive transcriptional activation from both PAX8- and PPAR-responsive promoters *(333)*. Further studies will be required to establish the mechanisms for each of these PPFP actions, especially under *in vivo* conditions, and which of these actions are most important for PPFP-mediated tumourigenesis.

3.2.2 Aims and Objectives:

PTCs show an overall survival rate exceeding 90% at 10 years. In contrast, FTCs are usually prone to blood-borne metastases, and carry a lower overall survival rate. Whereas FVPTCs, which generally show a slower growth pattern, are in some cases prone to lung metastases without any nodal metastasis. It was also observed that poorly circumscribed and widely invasive FVPTCs mimic the disease progress pattern of FTC, pushing the borders and signs of our classical understanding.

It has been shown in our previous study and supported by other literatures that FVPTC cases have a different prevalence of BRAF mutations compared to conventional PTCs, which are characterized by the occurrence of BRAF ^{V600E} (40-60%), whereas FVPTC cases have a much lesser prevalent (10%).

The PAX8-PPARγ fusion gene has been detected in a high percentage of FTC, FTC (16) and, later on, also in FVPTCs by some groups in different geographical locations (17–20).

In an attempt to find the prevalence of PAX8-PPARγ in our Indian patients, and to see whether or not, FVPTC cases share some of the molecular features of follicular tumours; we initiated the study with 62 thyroid neoplasia and 16 benign tissues. In addition we also investigated the association of this rearrangement with BRAF mutation and different clinico-pathological parameters.

3.2.3 Materials and Methods:

3.2.3.1 Tumour Tissue Specimens:

Frozen thyroid tissues from thyroid cancer patients who had attended the clinic between 2000 and 2010 were retrieved from the Tissue Repository of Tata Memorial Hospital, Mumbai, India for analysis of *PAX8/PPARγ* and *RET/PTC* gene rearrangements. A total of 62

malignant and 16 benign tissue specimens with adequate clinical and pathological information were studied. Neoplastic Tissue specimens included 32 PTC (26 Classical PTC and 6 FVPTC), 13 FTC, 6 FTA and 10 MTC.

Patients were staged using the tumour-node-metastases (TNM) system and classified according to the presence of extra-thyroidal extension, cervical nodes and distant metastases. All the analyzed tissues were snap frozen immediately in liquid nitrogen after the surgery and stored at -80^{o}C with RNA Latter (Sigma).

3.2.3.2 RNA Extraction and cDNA Synthesis from Thyroid Tissue:

RNA was extracted from the tissue using "HiPurA^{TM} Total RNA Miniprep Purification Spin Kit"(Himedia). Cryo-preserved tissue (approx 20-30 mg) was first crushed in liquid nitrogen. Liquid nitrogen was allowed to evaporate and before the tissue gets thawed 350-400 µL lysis solution containing 2-mercaptoethanol (1% v/v) was added and vortexed for 30 sec. Incubation of 15 to 30 min with intermediate vortexing is given for complete lysis. Clear tissue lysate was transferred to cleanup column (DBCA 01) and centrifuged at 10,000 rpm 2 min. The filtrate was collected and equal volume of 70 % ethanol was added. The mixture was applied on a RNA separation column (DBCA 02) and centrifuged at 10,000 rpm for 2 min. Then the column was centrifuged at 10,000 rpm with 350 µL of pre-wash solution for 15 sec. Filtrate was discarded and add 10 µL of 4U/µL of DNAase1 and 50 µL of 1X DNAase 1 digestion buffer was added and incubated for 30 min for on-column digestion of any DNA impurities. Another wash with 350 µL of pre-wash solution was applied. Thereafter, the column was given two consecutive washes with 500µL of wash solution at 10,000 rpm for 15 sec each. The column was eluted with 20µL DEPC treated elution buffer by centrifuging at 12,000 rpm for 1 min. Extracted RNA was immediately stored at -80 ^{o}C. The efficiency of extraction is checked by taking the absorbance ratio 260nm and 280nm (expected ratio 1.8-2.0)

cDNA was prepared by reverse transcription using the Revert Aid^{TM} First Strand cDNA Synthesis Kit (MBI Fermentas, #K1622). Appropriate amount of RNA (volume of RNA solution corresponding to 1µg) was taken and made up to 11.6 µL using sterile DEPC treated water. Random hexamer (1 µL) was added and incubated at 72 ^{o}C for 5 min. 7µL of master mix, containing 2µl of dNTP, 4µl of 5X buffer and 1µl of RNase inhibitor, was added and incubated at 25 ^{o}C for 5 min. Reverse transcriptase (0.4µL) was added and further incubated at 42 ^{o}C for 60 min and then at 70 ^{o}C for 10 min. The desired cDNA was stored at -80 ^{o}C *(335)*.

3.2.3.3 RT-PCR and Sequencing

Chromosomal translocation fusing the Paired Box gene (PAX8), and the nuclear receptor-peroxisome proliferator activated receptor (PPAR) was detected by reverse transcription PCR using cDNA synthesized from tissue sample.

Amplifications by PCR were carried out using forward primers F1 specific for PAX8 exon 6 and reverse primer R1 specific for PPARγ exon 1. The oligonucleotide primer sequences (Xcelris Labs, India) are presented in Table 3.3.1. RT- PCR was performed with 2µL cDNA, using the following conditions: 94 C for 30 sec, 60 C for 30 sec, and 72 C for 1 min for over 40 cycles of amplification. Reactions contained final concentrations of 20 mm Tris-HCl (pH 8.4), 50 mm KCl, 400µm dNTPs (Invitrogen), 2 mm $MgCl_2$, 10 pmol of both primers, and 1.5 U of Taq DNA polymerase (Invitrogen). Negative controls for cDNA synthesis and PCRs, in which the template was replaced by sterile water, were included in each experiment. RNA integrity and efficiency of cDNA synthesis were confirmed in each sample by RT-PCR for the housekeeping gene β-Actin. PCR products were analyzed by electrophoresis in a 5% polyacrylamide gel and purified by running in 1.5% Agarose gel. All results were repeated at least two times from different batches of cDNA at different times with same RNA sample.

Table3.2.1: RT-PCR primers for detection of PAX8-PPARγ fusion transcripts

Primer	Complementary Gene	Primer Sequence
F1	PAX8 (exon 6)	5'CGCGGATCCGCATTGACTCACAGAGCA3'
R1	PPARγ (exon 1)	5' CCGGAATTCGAAGTCAACAGTAGTGAA3'

Amplification of PAX8-PPAR cDNA variants containing PAX8 exons 1–6, 1–7, 1–7 plus 9, or 1–8, were expected to generate products of 296, 417, 519, and 606 bp, respectively (Fig. 3.2.3).

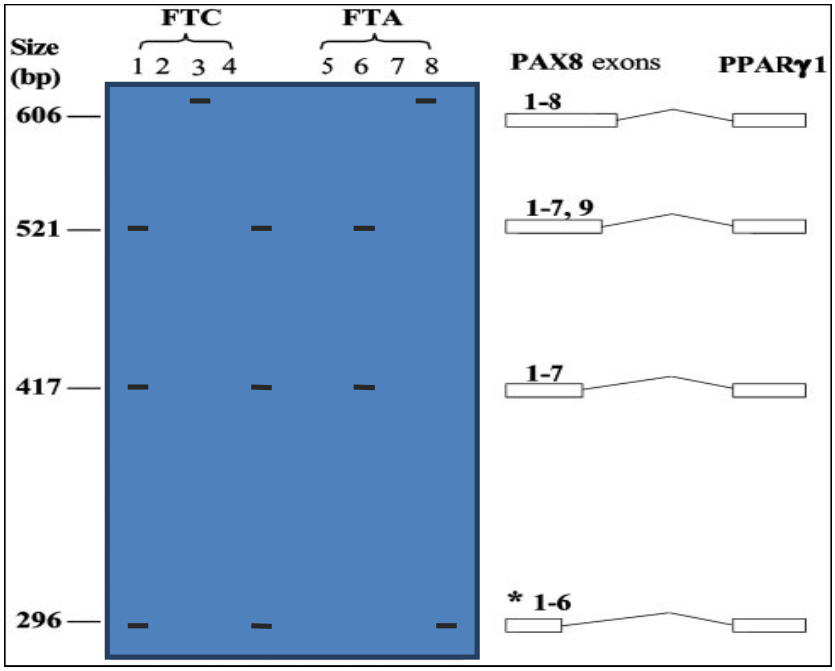


Fig 3.2.3: Schematic representation of PAX8-PPARγ fusion mRNA transcripts. Sizes of the fusion transcripts are shown on the left.

Patients' samples with rearrangements were purified and PCR based analysis is over and the amplicons obtained will be analysed by sequencing in both forward and reverse orientation

using the same primer set (ABI Prism 310 using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit version 2; Applied Biosystems, USA).

3.2.3.4 Silver Staining

After separation of the RT-PCR amplicons in 5% polyacrylamide gel, the gels were kept in 40:60 methanol:water for 30 min for fixing. Thereafter, the methanol-waterwas replaced with 2% Nitric acid solution and incubated for 10 min. The gel was thoroughly washed with distilled water to remove the traces of nitric acid. Subsequently, it was exposed to freshly prepared solution of 0.2% (200mg/100ml) Silver Nitrate in dark for 15 min. The gel was thoroughly washed with distilled water thrice for 30 sec each and was incubated in developer (20 ml chilled 3% sodium carbonate solution + 20 ml distilled water + 60μL of sodium thiosulphate + 40 μL of formaldehyde) till the bands were developed. The gel was washed with distilled water and 3% citric acid was added to stop the reaction. Finally the gel was stored in 80% methanol.

3.2.4 Results:

3.2.4.1 Prevalence of PAX8/PPARγ

In Tables 3.2.2, we have summarized the clinic-pathological and molecular data of the 62 thyroid carcinoma samples including 32 PTC (26 Classical PTC and 6 FVPTC), 13 FTC, 6 FTA, and 10 MTC.

The mean age ± SD was 47.6 ± 13.5 yr, and the mean tumour size ± SD was 3.9 ± 2.6 cm. By RT-PCR, we detected the PAX8/PPARγ rearrangement in 13 of the 62 cases analyzed (20.9%) (Fig. 3.2.4; Table 3.2.2). We confirmed the rearrangement, by FISH analysis in the two cases in which paraffin embedded tissue was available. In 16 cases the adjacent thyroid was analyzed by RT-PCR, and the PAX8/PPARγ rearrangement was not detected in any of these samples.

Table 3.2.2: Prevalence of *PAX8/PPARγ* Translocation in 62 thyroid neoplasia patients

Histology	Total	*PAX8/PPARγ*			
		Positive		Negative	
		N	%	N	%
Papillary Carcinoma	32	4	**12.5**	28	87.5
Classical Variant	26	2	**7.7**	24	92.3
Foll. Var. of PTC (FVPTC)	6	2	**33.3**	4	66.6
Anaplastic Carcinoma	1	0	-	1	100
Follicular Carcinoma	13	2	**15.4**	11	84.6
Follicular Adenoma	6	1	**16.6**	5	83.3
Medullary Carcinoma	10	2	**20**	8	80
Benign	16	0	-	16	-
Cell lines: ARO, NPA, FRO, WRO	4	0	-	4	-

Of the 62 samples studied rearrangements were found most prevalently in FVPTC (2 out of 6, 33%), followed by MTC (20%), FTA (16.6%) and FTC (15.4%). The rearrangement was not detected in the single case of ATC or in any of the four thyroid cancer cell lines.

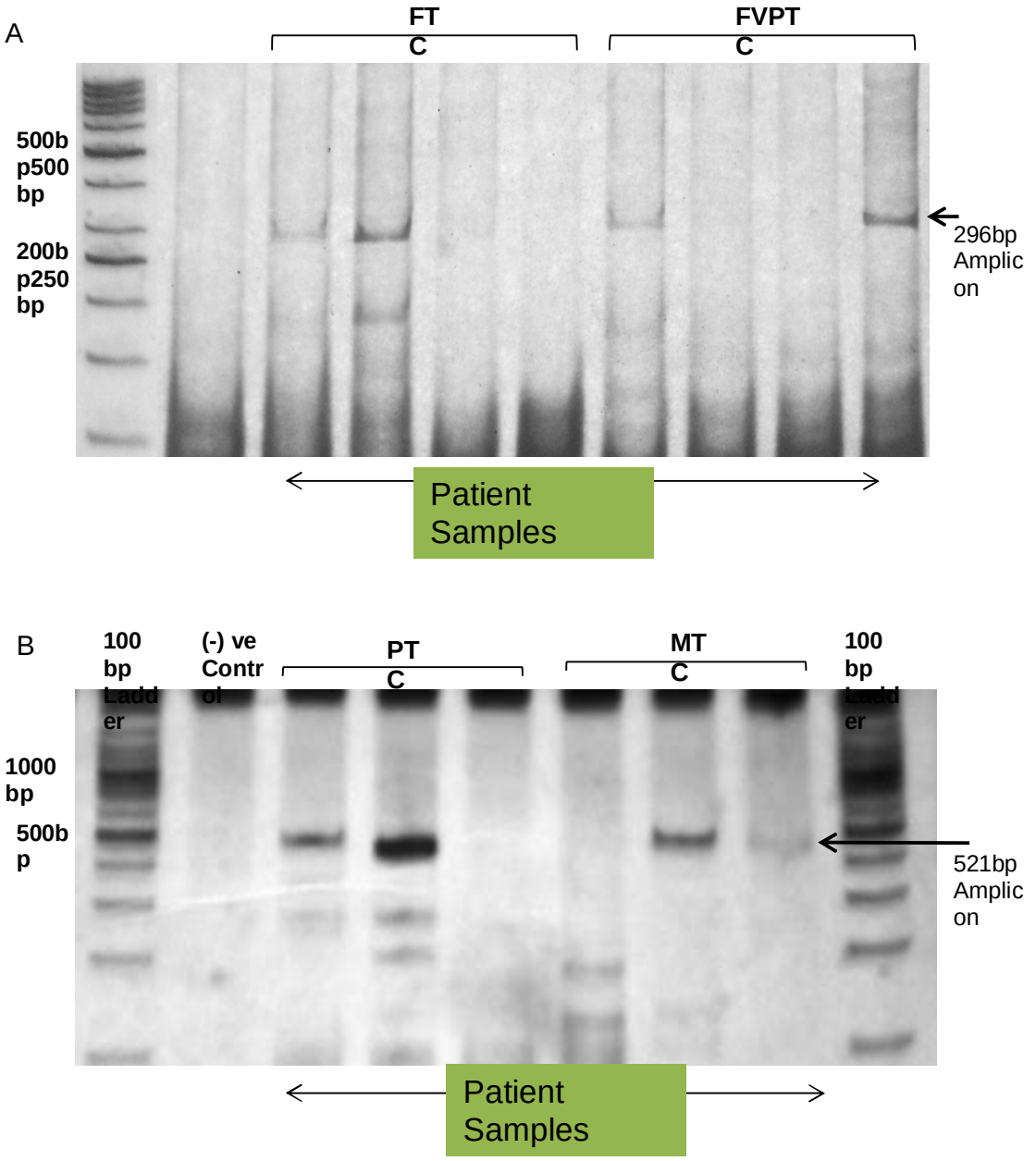


Fig 3.2.4: A and B, RT-PCR analysis of PAX8-PPARγ transcripts in thyroid neoplasias. The resulting DNA products were resolved by electrophoresis on a polyacrylamide gel and observed by silver staining. 296bp RT-PCR amplicons were observed in FCT and FVPTC (top) and 521bp amplicon was observed in PTC and MTC (bottom). DNA size markers indicating 100-bp and 50 bp intervals were used in lane 1 of each gel; negative control was run in lane 2 (top). A single PAX8-PPAR γ transcript is present for each case in panel.

Breakpoint analysis showed that both [Pax8 exon 1-6 to PPAR exon 1] and [Pax8 exon 1-7, 9 to PPAR exon 1] were equally present in our study group. PAX8 breakpoint at exon 6 is

found exclusively in FTC, FVPTC and FTA. Whereas, breakpoint at exon 7 and 9 are present only MTC and PTCs.

Table3.2.3: Association between *PAX8/PPARγ* mutation and clinico-pathological parameters in papillary thyroid carcinoma

	PAX8/PPARγ				Fisher's test (2 t)	Multivariate Analysis	
	Positive		Negative		*P*	*P*	Odds ratio (95% CI)
	N	%	N	%			
Tumour Stage (PTC)					0.014*	0.110	4.65 (0.70-30.7)
T I	0	0	17	100			
T II	2	8	23	92			
T IIIIV	6	31.5	13	68.4			
Gender (PTC)					0.482	0.437	2.13 (0.31-14.3)
Male	6	18.2	27	81.8			
Female	3	10.3	26	89.6			
Age(PTC)					1.0	0.7	0.648 (0.071-5.8)
≥45yr	7	15.9	37	84			
<45yr	2	11.1	16	88.8			
Metastasis (PTC)					0.028*	0.254	0.188(0.01-3.3)
Yes	7	26.9	19	73			
No	2	5.5	34	94.4			
Tumour size (mm) range, mean ± SD					0.176	0.360	2.24 (0.39-12.7)
<10 mm	0	0	8	100			
10–40 mm	3	9.6	28	90.3			
>40 mm	6	26	17	73.9			
Extra-thyroidal Extension					0.009*	0.05*	15.5 (0.98-245.8)
Yes	8	27.5	21	72.4			
No	1	3	32	96.9			

* p<0.05

3.2.4.1 Clinco-pathological Association:

The association between *PAX8/PPARγ* and various clinico-pathological parameters were assessed in 62 samples (Table 3.2.3) by Fisher's exact test, univariate and multitariate logistic regression analysis. The *PAX8/PPARγ* rearrangement was significantly associated with the presence of metastasis (P < 0.05), extra-thyroidal extension (P = 0.009) and size of tumours

(p=0.014). All but one the 9 patients with *PAX8/PPARγ* rearrangement had extra-thyroidal invasion. No significant association of *PAX8/PPARγ* rearrangement with age (p=1.0) and gender of patients (p=0.482).

Although Fisher's exact test showed association with three different clinico-pathological parameters, Multivariate regression analysis revealed existing association between ETE and *PAX8/PPARγ* [p=0.05, Odds ratio 15.5 (0.98-245.8) at 95% CI].

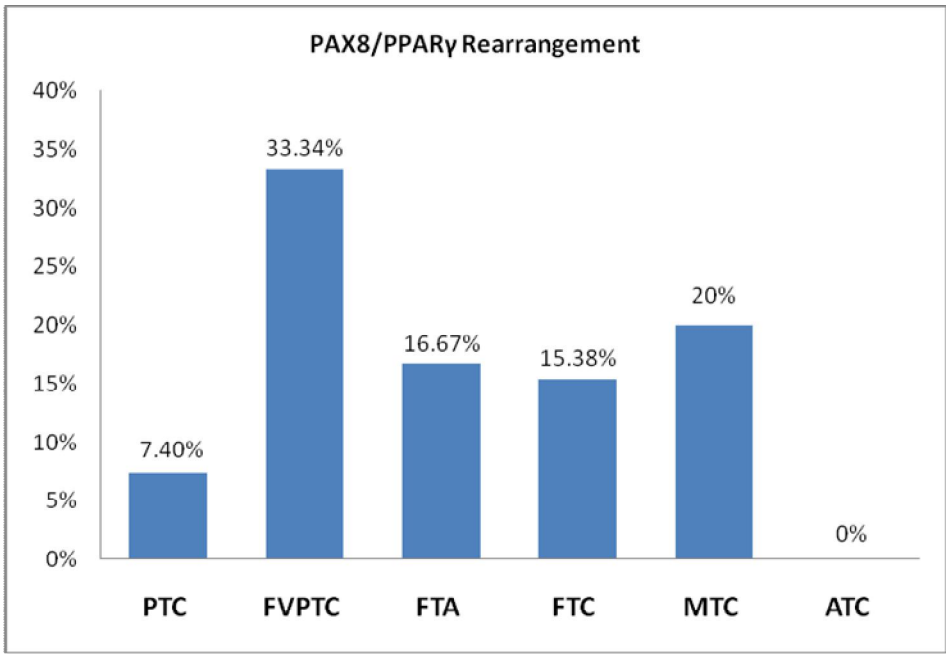


Fig 3.2.5: Prevalence of *PAX8/PPARγ* translocation in different histotypes of thyroid carcinoma

3.2.5 Discussion:

Our results raise some interesting issues on the putative relationship between FVPTC and FTC, and on the implications of the tumours' genotype for our understanding of their phenotype. The first issue concerns the much higher prevalence of *PAX8/PPARγ* in follicular variant of PTC (FVPTC) compared to classical variant of PTC. We report, for the first time

from India, in a cohort of 62 patients of thyroid carcinoma in which the *PAX8/PPARγ* fusion gene has been detected in a moderately high percentage of cases (20.9%).

Our preliminary data support the published data *(133)* and demonstrate that there is a association between the occurrence of *PAX8/PPARγ* rearrangement and the follicular growth pattern even in the setting of PTC also reported by Roque et. al. *(336)*. The nuclear features of FVPTC were of the PTC-type regardless of the presence or absence of *PAX8/PPARγ* rearrangement, thus supporting the assumption that such features probably reflect molecular alterations other than those related to the rearrangement *(337)*.

The significant association we have found between *PAX8/PPARγ* and extra-thyroidal invasiveness in the study group suggests that the rearrangement confers a higher invasive potential to the neoplastic cells in tumour microenvironment. These findings fit with those reported from other ethnicities of the world *(176)*.

Though our present study showed an association with lymph node metastasis (by Fisher's test), there was no association of rearrangement with distant metastasis. Since, a majority of patients in our series were not having distant metastasis, it was not possible to analyze the putative association of *PAX8/PPARγ* rearrangement with overtly invasive features of the tumours, as put forth in a previous study *(176)*.

The frequencies of *PAX8/PPARγ* in FTCs (15.4%) and FTAs (16.6%) are in accordance with the data on record *(338, 339)*. There was only one sample of PTC with coexistence of $BRAF^{V600E}$ mutation and the rearrangement. The patient presented with a binodal neck metastasis and ETE. Similar to the previous reports, no mutations in BRAF hot-spot coexisted with rearrangement in any of the follicular tumours *(283, 335, 340)*.

The issue concerning the putative relationship of FVPTC with conventional PTC and FTC needs to be addressed. In view of our data illustrating a high frequency of *PAX8/PPARγ*

rearrangement in FVPTC (33.3%) (Fig 3.2.5) in one hand and BRAF ^{V600E} in classical PTC (61.3%) on the other, it seems logical to conclude that some cases of the FVPTC do not share similar molecular features with papillary thyroid subtype (PTC).

Our conclusion is reinforced by the findings of Wreesmann et al., *(133)* who showed, using comparative genomic hybridization analysis, that the presence and pattern of genomic aberrations in FVPTC were significantly different from those in conventional PTC and similar to those observed in follicular tumours *(133)*. Therefore, it does not seem possible to rely upon histopathology for the identification of the subset of FVPTC that shows some of the molecular features of FTC. There is enough evidence now to rule out the idea that FVPTC, as a whole, should be considered as a subgroup of conventional PTC, and we propose that the metastatic pattern *(341-346)* and the molecular features described in the present study should be taken into consideration. This concept is represented schematically in Fig. 3.2.6.

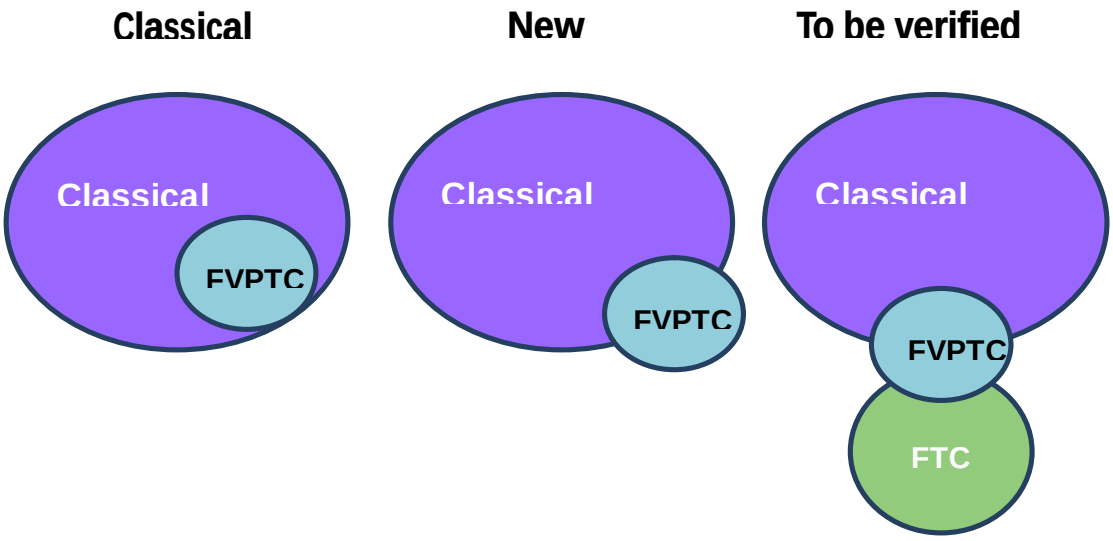


Fig 3.2.6: Schematic representation of the putative relationship between FVPTC and the two main histotypes of thyroid carcinoma (PTC and FTC).

The study of a larger series of FVPTC with a longer follow-up and detailed data on lymph node and lung metastases is necessary to find out the clinical significance of the occurrence of the three genetic alterations (*PAX8/PPARγ*, H, N, K RAS mutation and BRAF in association) in FVPTC.

3.2 RET PTC Rearrangement in Thyroid Carcinoma

3.3.1 Introduction:

RET is a single-pass transmembrane receptor tyrosine kinase (RTK) that is required for normal development, maturation and maintenance of several tissues and cell types. The developmental importance of RET is highlighted by a high degree of protein conservation in a range of species, from Drosophila through to Human. In the past several years, key studies have implicated that RET gene rearrangement is associated with cell proliferation, migration, invasion and survival of patients in several forms of cancer.

3.3.1.1 RET structure and function

The RET proto-oncogene lies in the pericentromeric region of chromosome 10q11.2 *(347)*. The expression of RET is regulated by DNA-binding factors that modulate basal transcription (such as SPI, SP3 and early growth response protein 1 (EGR1)) *(348)* and/or contribute to its tissue-specific expression patterns (such as SRY-box 10 (SOX10), paired box 3 (PAX3), NK2 homeobox 1 (NKX2 -1) and homeobox B5 (HOXB5)) by binding to upstream promoter and enhancer sequences *(349-351)*.

RET signalling is mediated by binding of a group of soluble proteins of the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs), which also includes neurturin (NRTN), artemin (ARTN) and persephin (PSPN) *(352)*. Unlike other RTKs, RET does not directly bind to GFLs, but it requires an additional co-receptor: that is, one of four GDNF family receptor-α (GFRα) family members, which are tethered to the cell surface by glycosyl-phosphatidy-linositol linkage. GFLs and GFRα family members form binary complexes that in turn bind to RET and recruit it into cholesterol-rich membrane sub-domains, which are known as lipid rafts, where RET signalling occurs *(352, 353)* (Fig 3.3.1). Upon binding of

the ligand coreceptor complex, RET dimerization and autophosphorylation on intra-cellular tyrosine residues recruits adaptor and signalling proteins to stimulate multiple downstream pathways.

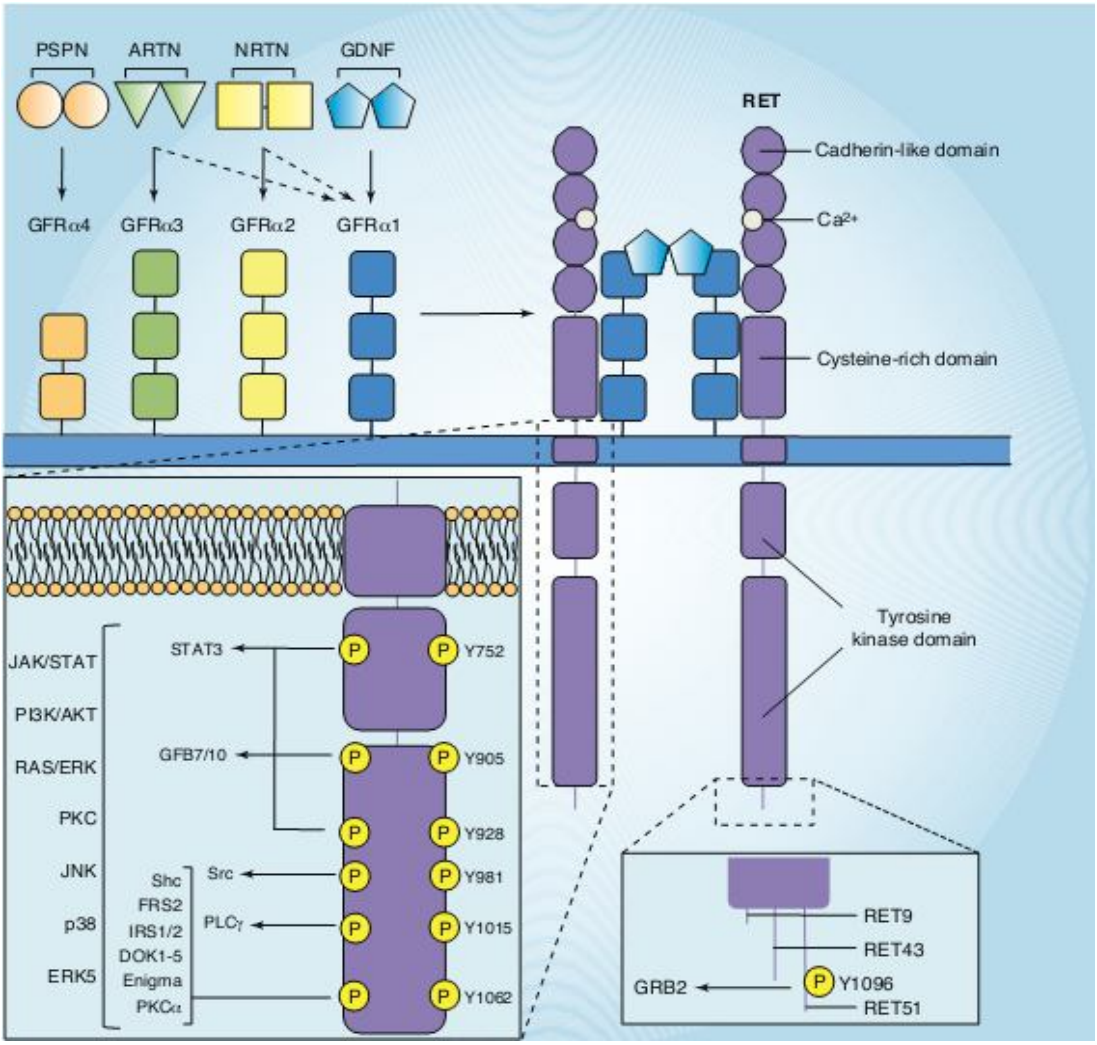


Fig 3.3.1: RET functional domains and downstream signaling. RET ligands bind their respective GFRa co-receptors (full arrows indicate the preferred interactions, broken arrow indicates potential interactions) and activate RET. GFRa proteins have three cysteine-rich domains (except for GFRa4, which has only two) and are attached to the plasma membrane by a GPI anchor. Activation of RET leads to trans-phosphorylation of tyrosine residues (Y) in the RET kinase domain resulting in the recruitment of different signaling molecules and adaptor proteins as highlighted in the zoomed area. (Source: Trends in Mol. Medicine, Volume 17, Issue 3, p149–157, March 2011, Cell Press)

3.3.1.2 RET rearrangements: RET and the other genes involved

RET/PTC rearrangement has been known for almost two decades as one of the most common molecular alterations in thyroid papillary carcinoma. It was discovered in 1987 by Fusco et al *(354)* using a transfection assay on NIH3T3 cells, which revealed the transforming activity of DNA isolated from papillary carcinomas. In the thyroid gland, RET protein is strongly expressed in parafollicular C-cells consistently with its role in the development of neural crest-derived structures *(354)*. In some PTCs, the tyrosine kinase domain of RET is fused with an heterologous gene that provides the promoter and the 5'coding region *(355)*. The product of this rearrangement is a chimeric oncogene named RET /papillary thyroid carcinoma (*RET/PTC*). Since the original report, at least 11 types of *RET/PTC* variants have been isolated *(355)*. Most of these rearrangements are between RET on chromosome 10 and genes located on different chromosomes. By contrast, *RET/PTC*1 and *RET/PTC*3 are intrachromosomal paracentric inversions because the genes involved H4 and RFG (also designated ELE1/ARA70/NCOA4) are also all located on chromosome 10 *(355,356)*. *RET/PTC*1and *RET/PTC*3account for the vast majority of the variants, while the others are very rare and have little clinical significance. The chimeric *RET/PTC* protein generated by the genetic recombinations lacks the transmembrane domain and the extracellular regulatory region of RET but retains the coiled-coil domains in the RET partner coding sequences, necessary for the dimerization process. This determines the cytoplasmic location of the chimeric protein, its ligand-independent dimerization, and its autophosphorylation at residues 1015 and 1062 responsible for the oncogenic activity of the *RET/PTC* chimera *(357)*.

3.3.1.3 *RET/PTC* in thyroid cancer

RET rearrangements are an exclusive occurrence of thyroid gland. A possible explanation for this restriction is that contiguity between RET and the recombinant genes occurs in nuclei of

thyroid cells but not in other cell types *(358)*. After its initial discovery in 1985, a large survey conducted in the following decades evidenced the frequent occurrence of *RET/PTC* in PTC and its sporadic oligoclonal occurrence in benign thyroid lesions. The estimated prevalence of RET rearrangement in PTC is highly variable among different studies. A crucial factor responsible for this wide variability is the detection method. Another important factor is tumour subtype composition. PTC is a heterogeneous tumour including very frequent variants such as classic PTC, FVPTC, and some uncommon PTC variants such as tall-cell PTC (tcPTC), diffuse sclerosing, columnar-cell, Hurthle-cell, cribri-form, and solid variants *(359)*. The reported prevalence of RET rearrangements in classical PTC was generally higher than that in FVPTC. *RET/PTC* , mostly the *RET/PTC*1, has been detected in a relevant percentage of Hurthle-cell variant PTC, in the cribriform variant, which is typically associated with familial adenomatous polyposis, and in hyalinizing trabecular tumour, a borderline thyroid neoplasm with many histological features similar to PTC *(360, 361)*. Ionizing radiations are known to be a relevant cause for genetic alteration and carcinogenesis because they are particularly effective in inducing DNA double-strand breaks. The association of *RET* rearrangements with radiation exposure was sustained by the observation that the incidence of PTCs is severely increased in radio-iodine contaminated areas following a linear relationship with dose exposition, as seen in Chernobyl. *(362)*. In the comparative analysis of *RET* rearrangements in sporadic and radiation-induced thyroid tumours performed by Nikiforov et al. *(363)*, *RET/PTC*1 was shown to be the dominant type within sporadic carcinomas and was strongly related to the classic variant, while *RET/PTC*3 was predominant among radiation-induced carcinomas and more frequent in the solid PTC variant (Fig 3.3.2).

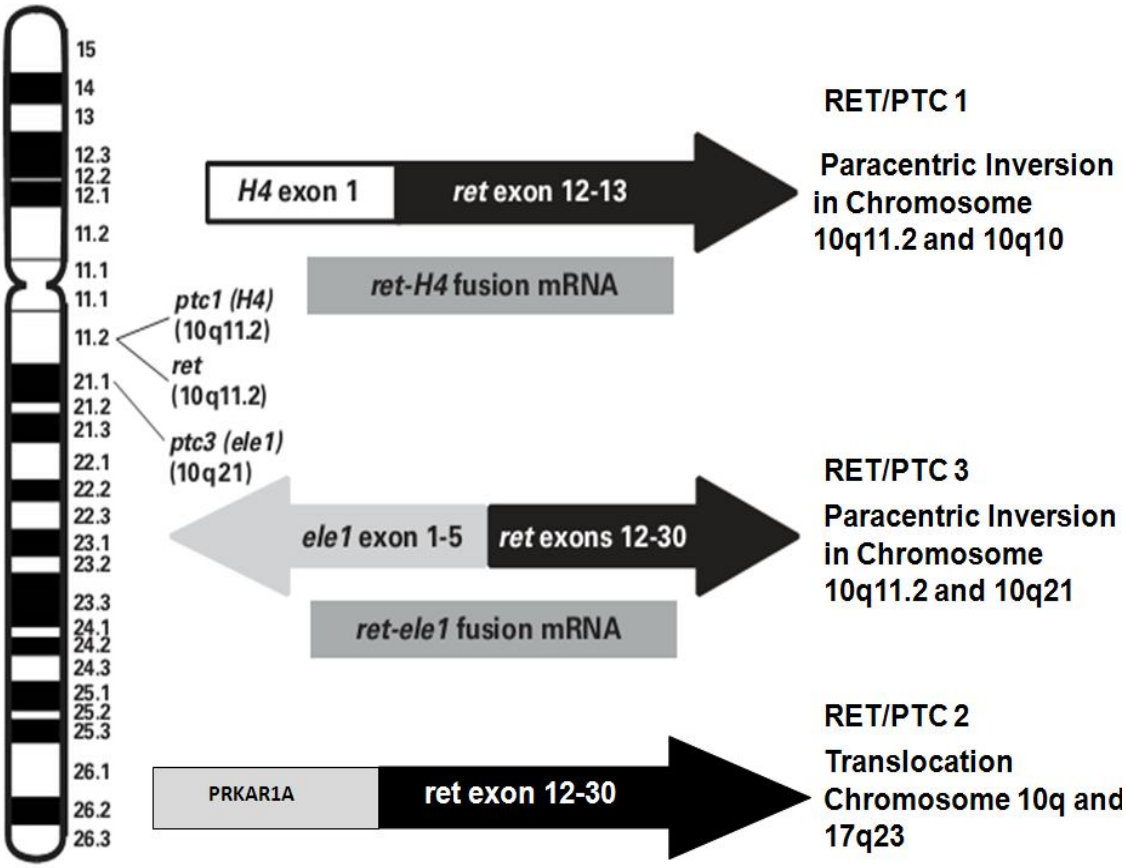


Figure 3.3.2: Schematic representation of the three initially described types of *RET/PTC* (*RET/PTC*1, *RET/PTC*2, and *RET/PTC*3). All rearrangements result from the fusion of the tyrosine kinase domain of *RET* to the 5' portion of different genes that are constitutively expressed in thyroid follicular cells. All *RET* fusion genes encode putative dimerization domains (CC, coiled-coil domain; C18 and C39, cysteine residues forming disulfide bonds during dimerization) essential for ligand-independent activation of the truncated *RET* receptor.

3.3.1.4 Patho-physiological aspects of *RET/PTC*

There are several lines of evidence pointing to *RET/PTC* as a driving event in thyroid carcinogenesis. The rat thyroid cells- PCCl3 change morphology, lose their differentiated functions, and are no longer TSH dependent for growth when stably transformed by *RET/PTC*3 *(364)*. However, acute expression of *RET/PTC* is sufficient to block the expression of TSH receptor and generation of cAMP, but it is insufficient to allow cells to

grow in the absence of TSH, suggesting that *RET/PTC* is a weak tumour-initiating factor and that secondary genetic or epigenetic changes are required for full transformation into a cancer cell as suggested by Wang et.al. *(365)*. Transgenic mice expressing *RET/PTC*1 develop thyroid papillary carcinomas with features very similar to those shown by human PTC. These tumours are characterized by nuclear grooves and ground glass cells, slow growth rate, TSH responsiveness, continued expression of thyroglobulin, and loss of radioiodide concentrating activity *(365, 366)*. Similarly, *RET/PTC*3 transgenic mice develop thyroid hyperplasia and locally invasive solid subtype of PTC with lymph node metastasis *(367)*. However, not all the *RET/PTC* transgenic animals develop thyroid cancer, and it appears only after a long latency period. This evidence suggests that other molecular events are required for the development of thyroid carcinomas. This concept is further supported by the finding that *RET* rearrangements can occur only in a fraction of the cells in some PTC, thus being a secondary event in these tumours, and by the presence of *RET/PTC* in sporadic cells in HT and benign nodules. Radiation exposures, cellular over-proliferation, production of free radicals and chemokine secretion have been hypothesized to account for the occurrence of intrachromosomal rearrangements in these benign thyroid lesions *(368)*. The biological significance of *RET/PTC* in benign lesions is a challenging task. A recent study has tried to answer the question whether this oncogene changes the fate of benign nodules. Sapio et al. *(369)* searched for *RET* rearrangements in a cohort of benign thyroid nodules in patients subjected to a careful clinical and ultrasonographic follow up for 3 years. The researchers did not find evidence of cancer development but demonstrated that nodules harbouring *RET/PTC* grow more rapidly than those without the oncogene. This finding is consistent with the hypothesis that even the presence of a small portion of cells having *RET/PTC* may have biological and clinical consequences in benign nodules. While the mechanisms underlying the more rapid growth of these nodules remain unclear, it can be hypothesized that the pro-

inflammatory transcriptional program activated by *RET/PTC*, including upregulation of various cytokines, chemokines, and their corresponding receptors, can stimulate thyroid cell proliferation through a autocrine/paracrine mechanism extended to the neighboring cells and involving a larger part of the nodule as reported by Mellilo et.al., and Borrello et. al. *(370, 371)*. More studies with longer follow-up are needed to clarify the clinical significance of low level of *RET* rearrangements in benign nodules.

3.3.2. Aims and Objectives

As inconclusive cytology from a FNAB is seen quite frequently and BRAF as molecular marker does not cover the complete PTC histotype. Hence, the introduction of additional molecular markers in the preoperative diagnosis of thyroid nodules has been proposed in recent years. In this study, we explored the clinical implications of preoperative detection of rearrangements of the *RET* gene in thyroid nodules. The prevalence of *RET/PTC* in PTC depends on the histological subtypes, geographical factors, radiation exposure, and detection method.

Though initially reported decades ago, there are relatively very few reports of this rearrangements and its prevalence in different races of the world. Recently, very sensitive detection methods, FISH and Southern blot on RT-PCR amplicons, demonstrated increased frequency of detection of *RET* rearrangement.

Our study is the first of its kind from patients from Indian population aimed towards detection of *RET/PTC* rearrangements in PTC and study its coexistence with BRAF point mutations along with its clinico-pathological significance.

3.3.3 Materials and Methods:

Identical set of frozen tissue samples were used for *PAX8/PPARγ* and *RET/PTC* rearrangement.

3.3.3.1 Detection of *RET/PTC* Rearrangement by RT-PCR:

As recent literature reports suggest that roughly 90% of *RET/PTC* rearrangements observed in thyroid tumours are *RET/PTC*1and *RET/PTC*3, while *RET/PTC*2 is rarely seen (Fig 3.3.2). Therefore, our study was focused on these three variants. Previous reports suggest the prevalence of these rearrangements in PTC. However, as we are reporting for the first time from India, we have included all the histological subtypes as per their prevalence.

RET/PTC rearrangements were detected by RTPCR with the same cDNA used in PAX8/PPARγ translocation analysis. RNA extraction and cDNA synthesis was performed as described previously in Section 3.1.3.

Forward primers were designed on the coiled-coil domains of the *RET* fusion partners: H4 (10q21) for *RET/PTC*1, RLα for *RET/PTC*2 (17q23) and RFG for *RET/PTC*3 (10q11.2). The common antisense primer was specific for *RET* gene.

Amplifications by PCR were carried out using forward primers specific for H4, RIα and ELE1 and reverse primer specific for *RET* gene in three separate PCR reactions for each sample. The oligonucleotide primer sequences (Xcelris Labs, India) are presented in Table 3.3.1. RT- PCR was performed with 2µL cDNA, using the following conditions: 94 ºC for 15 sec, 60 ºC for 30 sec, and 72 ºC for 1 min for over 40 cycles of amplification.

Reactions contained final concentrations of 20 mm Tris-HCl (pH 8.4), 50 mm KCl, 400um dNTPs (Invitrogen), 2 mm MgCl2, 10 pmol of both primers, and 1.5 U of Taq DNA polymerase (Invitrogen). Negative controls for cDNA synthesis and PCRs, in which the template was replaced by sterile water, were included in each experiment. Positive controls were tumours samples harbouring *RET/PTC* rearrangements. RNA integrity and efficiency of cDNA synthesis were confirmed in each sample by RT-PCR for the housekeeping gene β-Actin. PCR products were analyzed by electrophoresis in a 5% polyacrylamide gel and

purified by running in 1.5% Agarose gel. All results were repeated at least two times from different batches of cDNA at different times with same RNA sample.

Table 3.3.1: RT-PCR Primers for detection of RET/PTC Rearrangements:

Primer	Complementary Gene	Primer Sequence	Amplicon Size
RETPTC1F	H4	5'CAAAGCCAGCGTTACCATCG3'	81 bp
RETPTC2F	RIα	5'GAAATTGTGGGGCATCGACC3'	108 bp
RETPTC3F	ELE1 (RGF)	5'CAAGCTCCTTACATACC3'	134 bp
RETPTC R	RETPTC	5'CCTTCTCCTAGAGTTTTTCC3'	why blank?
B Actin F	β Acitn	5' TGC GTG ACA TTA AGG AGA AG3'	
B Actin R	β Acitn	5' GCT CGT AGC TCT TCT CCA 3'	

3.3.4 Results:

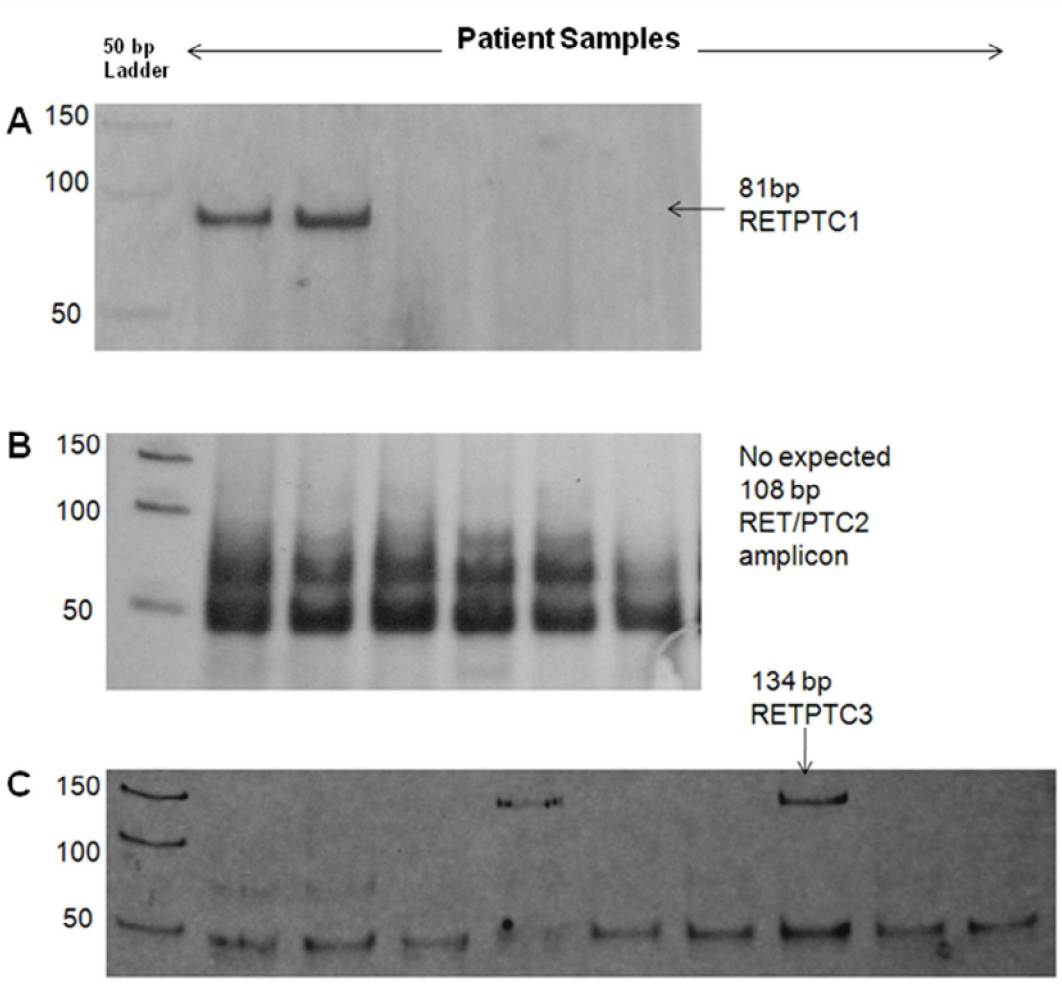


Fig 3.3.3: A, B and C showing RT-PCR analysis of RET/ PTC1, RET/ PTC2 and *RET/PTC*3 expression in representative thyroid tumour samples. RET/ PTC1 (A) and *RET/PTC*3 (C) shoed positive amplicons for the two rearrangements; whereas RET/ PTC2 amplicon was not found in any sample indicating absence of the chimeric transcript in the tissues.

Table 3.3.2: Prevalence of RET PTC 1rearrangement in 62 patients with thyroid neoplasia

Histology	Total	RET PTC1			
		Positive		Negative	
		N	%	N	%
Papillary Carcinoma	32	9	28.1	23	71.8
Classical Variant	25	9	36	16	64
Poorly Differentiated (PDTC)	0	0	0	0	0
Foll. Var. of PTC (FVPTC)	6	0	0	6	100
Tall Cell Variant	0	0	0	0	
Hurthle Cell Carcinoma (HCC)	1	0		1	100
Diffuse Sclerosing variant	0	0		0	
Follicular Carcinoma	13	0		13	100
Follicular Adenoma	6	0		6	
Medullary Carcinoma	10	0		10	
Benign hyperplasia	16	0		16	

The second most frequent genetic alteration found in PTC is RET rearrangement leading to the generation of chimeric *RET/PTC* oncogenes. To confirm the expression of fusion mRNAs in tumour tissues, assay was performed with primers designed to flank the fusion point between *RET* and its partner gene (H4 or RFG) as illustrated in Fig. 3.3.2 and 3.3.3. Amplification of *RET* gene revealed two specific translocation, *RET/PTC*1 *(H4-RET)* and *RET/PTC*3 *(RFG-RET)* in our patient population. Whereas *RET/PTC2* rearrangements was

not found. To further confirm whether *BRAF* mutation and *RET/PTC* rearrangements are coexisting or alternative events, prevalence of *BRAF* ^{V600E} was also investigated.

Table 3.3.3: Prevalence of *RET/PTC3*rearrangement in 62 thyroid neoplasia patients

Histology	Total	RET PTC3			
		Positive		Negative	
		N	%	N	%
Papillary Carcinoma	32	9	28.1	23	71.8
Classical Variant	25	8	32	17	68
Poorly Differentiated (PDTC)	0	0	0	0	0
Foll. Var. of PTC (FVPTC)	6	1	16.6	5	83.3
Tall Cell Variant	0	0	0	0	
Hurthle Cell Carcinoma (HCC)	1	0	-	1	100
Diffuse Sclerosing variant	0	0	-	0	
Follicular Carcinoma	13	0	-	13	100
Follicular Adenoma	6	0	-	6	
Medullary Carcinoma	10	0	-	10	
Benign hyperplasia	16	0	-	16	

In Tables 3.3.1, 3.3.2 and 3.3.3 we have summarized the prevalence and correlation with clinico-pathological data of the 62 cases of thyroid carcinoma including 32 PTC (26 Classical PTC and 6 FVPTC), 13 FTC, 6 FTA and 10 MTC.

The mean age ± SD was 47.6 ± 13.5 yr, and the mean tumour size ± SD was 3.9 ± 2.6 cm. *RET/PTC* rearrangement was found in 14 (22.5%) of 62 thyroid carcinoma patient samples.

Among all the histological subtypes, *RET/PTC* rearrangement was exclusively found in PTC and not in any other subtype including FTC, MTC or FTA. Rearrangement was detected in 13 (52%) of the 25 classical PTCs and one (16.7%) of six FVPTC samples. One case of PTC with Hurthle Cell pattern did not show any mutation.

Table 3.3.4: Prevalence of *RET/PTC* Rearrangement along with its complementarities and coexistence with BRAF^{V600E} mutation in PTC

Mutation	*RET/PTC* (n/N)	PERCENTAGE
***RET/PTC* (1, 2 and 3)**	**14/32**	**43.7%**
***RET/PTC* 1**	**9/32**	**28%**
***RET/PTC* 2**	**0/32**	**-**
***RET/PTC* 3**	**9/32**	**28%**
Coexistence of RETPTC and BRAF:		
***RET/PTC*1 + BRAF**	**5/32**	**15.6%**
***RET/PTC*3 + BRAF**	**9/32**	**28%**
***RET/PTC*1+ RETPTC3+BRAF**	**3/32**	**9.3%**
Complementarities in RETPTC and BRAF:		
***RET/PTC* ONLY**	**4/32**	**12.5%**
BRAF ONLY	**7/32**	**21.8%**

Both *RET/PTC*1 and *RET/PTC*3 were equally prevalent with nine cases (28.1%) each. RET/PTC1 was found in 9 (36%) of 25 cases of classical PTC exclusively. Whereas, *RET/PTC*3 was found in 8 (32%) of 25 PTC and 1 (16.7) of 6 FVPTCs.

Relevantly, taken together, BRAF and *RET/PTC* showed a considerable coexistence as well as occurring independently. $BRAF^{V600E}$ mutation was found in all the 9 (28% of the PTC) RET/PTC 3 rearrangement (Fig 3.3.4). Whereas *RET/PTC* 1 was detected to coexist with *BRAF* in 5 (15.6% of PTC) Patients. Three rearrangement in combination was found in 3 (9.3%) of 32 PTC. *RET/PTC* alone represents 12.5 % and *BRAF* represent 53.4% of PTC population. Thus, taken together, *BRAF* and *RET/PTC* detection will help to identify 65.9% (12.5% +53.4%) of PTC samples in our population.

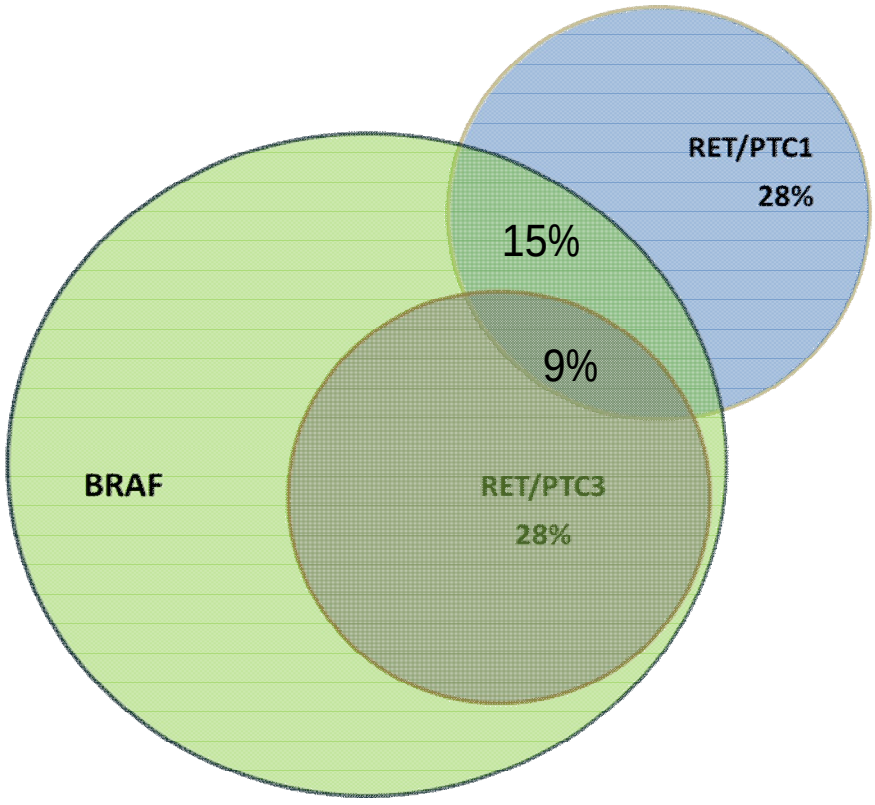


Fig 3.3.4: Coexistence of *RET/PTC* and *BRAF* point mutation in Papillary Thyroid Carcinoma.

3.3.3.2 Clinico-pathological correlation:

The association between *RET/PTC* and various clinico-pathological parameters were assessed in 62 samples (Table 3.3.3) by Fisher's exact test, univariate and multitariate logistic regression analysis. There was a considerable coexistence of *RET/PTC*3 and *BRAF* in the study group ($p<0.001$). Although there was no association of *RET/PTC* (1 and 3) rearrangement with extra-thyroidal extension, metastasis, stage, tumour size, age at diagnosis or gender.

3.3.5 Discussion:

Our understanding of the molecular biology of PTC has made a step forward with the discovery that roughly 50% of PTC harbour one specific activating point mutation in the *BRAF* gene *(179, 191, 218, 372)*. Though *BRAF* is an attractive molecular marker with high prevalence combined in PTC, there is an urgent need for finding additional markers for remaining 50% PTC population harbouring no *BRAF* mutation.

Another genetic lesion that is often present in PTC is the recombination of the RET kinase to heterologous genes, leading to the generation of chimeric *RET/PTC* oncogenes *(373)*. There is considerable variation in prevalence of *RET/PTC* rearrangements in published reports from different ethnicities. Though its occurrence is lesser than that of *BRAF* mutations, there is a controversy on its role in thyroid tumourigenesis and coexistence with *BRAF*. Incidental detection of *RET/PTC* in Hashimoto thyroiditis also complicate the use of *RET/PTC* in the molecular diagnosis of PTC *(374-377)*.

In this study, we also analyzed the 62 samples for the presence of either *RET/PTC*1, *RET/PTC*2 and *RET/PTC*3 and found that 43.7% of PTC samples were *RET/PTC* positive. In contrary to previous reports, 28 % patients harbouring *RET/PTC* were positive for *BRAF*

(286, 314). All of these samples with coexisting genetic alterations *RET/PTC*3 rearrangement. This limits the use of *RET/PTC*3 rearrangement as additional diagnostic marker for PTC. However 12.5% PTCs analyzed were exclusively carrying *RET/PTC*1 rearrangement giving us an advantage to use it as a diagnostic marker.

Table 3.3.5: Association between RET PTC rearrangements and clinico-pathological parameters in papillary thyroid carcinoma

	RET PTC (1,2,3)				Fisher's test (2 t)	Multivariate Logistic Regression Analysis	
	Positive		Negative		*P*	*P*	Odds ratio (95%CI)
	n	%	n	%			
Tumour Stage (PTC)					0.365	0.708	0.693 (0.12-4.7)
T I	2/9	22.2	7	77.7			
T II	6/11	54.5	5	45.4			
T IIIIV	6/12	50	6	50			
Gender (PTC)					1.0	0.729	1.4(0.20-9.6)
Male	7/16	43.7	9	56.2			
Female	7/16	43.7	9	56.2			
Age(PTC)					0.711	0.204	3.7 (0.49-27.8)
≥40yr	10/21	47.6	11	52.4			
<40yr	4/11	36.3	7	63.6			
Node Metastasis					0.307	0.715	1.63 (0.12-22.2)
Yes	9/47	74.4	8	25.6			
No	5/39	28.2	10	71.8			
Tumour size (mm) range, mean ± SD					0.812	0.626	1.3 (0.37-5.1)
<10 mm	2/6	33.3	4	66.6			
10–40 mm	6/13	46.1	7	53.8			
>40 mm	6/13	46.1	7	53.8			
***BRAF*^{V600E} Mutn.**					**0.0731**	0.097	5.4(0.73-39.5)
BRAF Positive	9/13	69.2	4	30.7			
BRAF Negative	6/18	33.3	12	66.6			
Extra-thyroidal Extension					0.412	0.851	1.33 (0.06-26.8)
Yes	12/24	50	12	50			

Thus, we envisage the possibility that the *BRAF* mutation analysis could be combined with *RET/PTC* detection to increase the fraction of identifiable PTC. A larger and prospective

study will be necessary to calculate the diagnostic utility of this dual marker analysis in FNAB samples to avert unwanted surgery in benign hyperplasia. Of course, the absence of *BRAF* mutations will not exclude a malignant condition *(378)*. Nonetheless, a positive finding can support decision making about the extent of surgery, planning for radioiodine therapy and probable chemotherapy more informed.

Another challenge in cytology, faced in diagnosis of FVPTC needs to be addressed in this context. Our data supported by previous reports, show a low prevalence of *RET/PTC* (16%) *(355)* and *BRAF* (11.7% as described in chapter 2.4) in FVPTC *(224, 379, 380)*. This means that a large fraction of PTC-FV would remain unrecognized at the molecular analysis with only *RET/PTC* and *BRAF*. Therefore, there is need for novel molecular markers like PAX8/PPARγ (33.3% as reported by us in chapter 2.4) for specific FVPTC subtype that can be used in adjunct to *RET/PTC* and *BRAF* detection.

Finally, multiplexing of all these markers in molecular diagnosis can provide an advantage in determining treatment modality and better prognosis for thyroid cancer patients, particularly in our country, where access to molecular techniques is available in limited centres.

Chapter 4

RET gene point mutations in Medullary Thyroid Carcinoma

4.1 Introduction

4.1.1 Medullary Thyroid Carcinoma: MTC is a rare type of cancer constituting only 5–10% of thyroid cancer cases. Of the 54,000 cases of thyroid cancer reported by Horner et.al. from the National Cancer Base in US, from 1985 to 1995, 2,000 are MTC *(381)*. Seventy-five percent of MTCs are sporadic; while the rest are associated with hereditary germ-line mutations in RET proto-oncogene *(382, 383)*. MTC can also coexist with other neoplasia, constituting the syndromes like MEN-2A and MEN-2B. Following anaplastic thyroid carcinoma, MTC has the second worst prognosis with an average 10-year survival being 50–80%, but this rate varies depending on age and TNM stage at the time of diagnosis *(384-387)*. Unfortunately, half of the MTC patients present with metastases at first diagnosis. Like other thyroid cancers, frequency of MTC is higher in women, with a female to male ratio of 2:1 *(388)*.

4.1.2 Histology of MTC:

Parafollicular cells constitute only 0.1% of thyroid cells. They are scattered throughout the thyroid gland, but are most numerous at the junction of the upper third and lower two thirds. Because they are responsible for synthesizing, storing, and secreting calcitonin, parafollicular cells possess extensive endoplasmic reticulum; this endoplasmic reticulum stains clear and therefore parafollicular cells are also referred to as "clear cells" or "C-cells" *(389)*. Follicular cells develop as early as the 3rd week of gestation, and are derived from a diverticulum of the foregut, whereas parafollicular cells develop around the 12th week of gestation, and are derived from neural crest tissue.

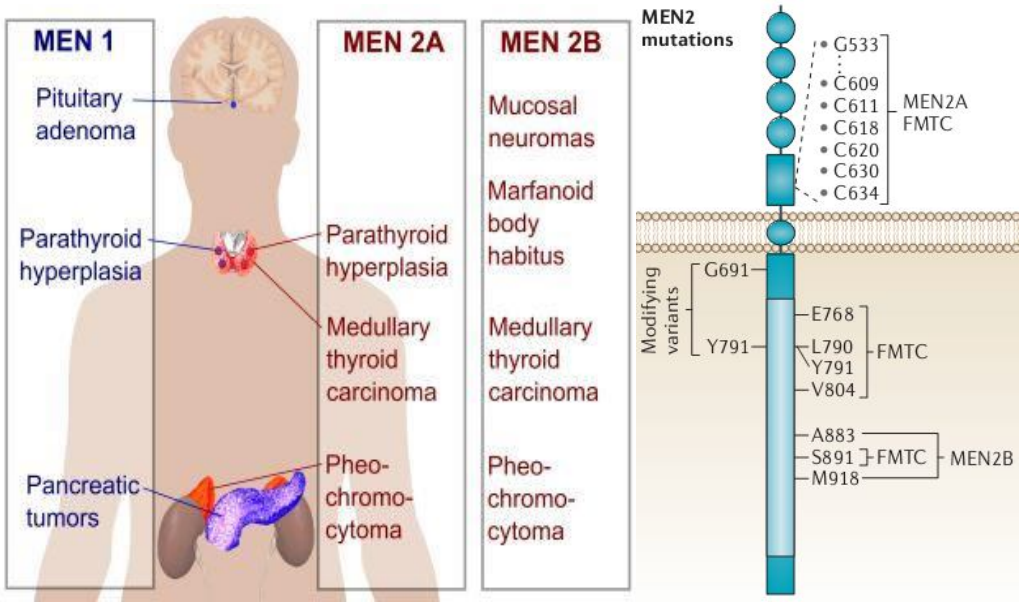


Fig 4.1: A. MTC is associated with multiple endocrine neoplasia syndrome, a group of medical disorders associated with tumours of the endocrine system. MEN includes MEN2A, MEN2B and familial medullary thyroid cancer, each with its own characteristic pattern. **B**. Oncogenic heritable RET mutations that are identified in patients with multiple endocrine neoplasia type (MEN) are generally point mutations that lie at specific sites in the RET protein. (Source: http://en.wikipedia.org/wiki/Multiple_endocrine_neoplasia)

This embryologic dichotomy has implications for the development of MTC and its unique treatment as a neuroendocrine tumour.

4.1.3 C-Cell Hyperplasia (CCH)

In normal thyroid tissue, C-cells are only seen sporadically around the thyroid follicle. When C-cells proliferate at an accelerated rate it results into CCH. Histologically, CCH is defined by the presence of more than six C-cells per thyroid follicle, or more than 50 C-cells visualized in one low power field (100×) *(391-394)*.

Frank MTC is always preceded by CCH; but the the presence of CCH usually, not always, implies progression to MTC *(53, 395)*. In fact, 20–30% of healthy individuals may have CCH evident on thyroid biopsy, and CCH is also commonly seen in autoimmune

thyroiditis and FTCs. When C-cells become prolific, they are more likely to disrupt and invade the follicular basement membrane. Disruption of the basement membrane is what defines MTC from CCH *(391, 396, 397)*. The extent of CCH and rate of progression to MTC are associated with the type of RET mutation, which is discussed more thoroughly in the upcoming pages.

4.1.4 Pathology:

Grossly, MTC is firm in consistency and gray-white to tan-pink in color; it is well circumscribed but not encapsulated. Microscopically, MTC consists of many cells separated by fibrous stroma *(53, 395)*. Unlike normal parafollicular cells which are usually round, MTC cells can be polygonal, round, or most often, spindle shaped. Because of their extensive endoplasmic reticulum for calcitonin production, the cytoplasm stains eosinophilic with conventional H&E staining; likewise, the cells also stain positive for calcitonin and carcinoembryonic antigen (CEA) on immunohistochemical staining. Extensive calcification and amyloid deposition also help distinguish the tumour. Mitotic figures are rarely seen.

Parafollicular cells primarily secrete calcitonin. Other secreted products include CEA, somatostatin, proopiomelanocortin (POMC), ACTH, vasoactive intestinal peptide (VIP), gastrin-releasing peptide, neurotensin, prostaglandins, kinins, serotonin, histaminase, chromogranin A (CgA) [especially in patients with concomitant pheochromocytoma], and neuron-specific enolase *(398-401)*. Patients with elevated calcitonin and/or VIP levels may present with diarrhea and flushing, and patients with elevated ACTH levels may present with typical Cushingoid signs and symptoms.

4.1.5 RET mutations and hereditary medullary thyroid cancer (HMTC):

To date, 98% of affected families with HMTC apparently exhibit genetic linkage to the RET gene locus and only a small percentage of MEN2 families have had no RET mutation detected (Fig 4.1). Isolated FMTC has been traditionally associated with germline activating mutations of the extracellular region of RET, mainly at exons 10 and 11, in the extracellular domain which is associated with the three-dimensional ligand binding pocket. These mutations lead to ligand-independent dimerization and receptor activation *(402,403)*. Non-cysteine mutations of the intracellular region of RET in exons 13–16 are less commonly linked to FMTC while mutations in other exons have been rarely reported in isolated families. Some mutations (particularly codons 532, 533, 630, 769, V804M, 844, 912) are thought to be relatively specific for FMTC *(404)*. However, codon 533 is also associated with MEN2A, indicating that only time and observation in large numbers of families can confirm this specificity. Nowadays, FMTC constitutes a challenging form of MTC, which is considered as a phenotypic mildest variant of MEN2A. A number of FMTC patients finally have MEN2A while a significant overlap in the observed RET mutations, is commonly found. Different RET mutations lead to the distinct clinical syndromes of MEN2A, MEN2B, and FMTC while a significant overlap exists between RET mutations associated with FMTC or MEN2A. The great majority of patients with MEN2A have mutations of RET in exons 10, 11, 13-16 while patients with MEN2B exhibit a single mutation at exon 16 *(405, 406)*. Due to a strong genotype-phenotype correlation in MTC, the genetic analysis and the identification of specific germline RET mutations offer important information regarding the penetrance of MTC and associated lesions. In addition, the same mutations are associated with significantly earlier progression from C-cell hyperplasia to MTC and earlier lymph node involvement than patients with most other mutations related to MEN2A and FMTC *(407, 408)*.

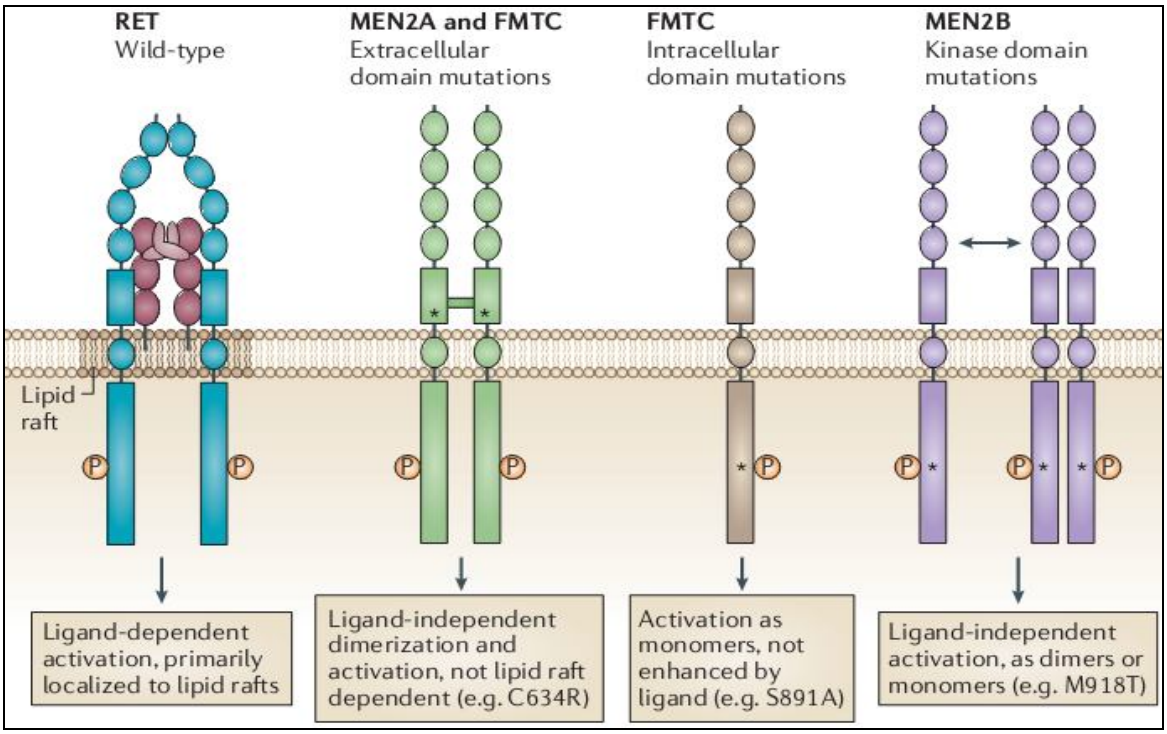


Fig 4.2: Molecular mechanisms of oncogenic RET mutations. The activation of wild-type RET requires the binding of a ligand and co-receptor complex. However, mutant forms of RET identified in human cancer can have one of several different activation mechanisms. (Source: and Nature Review, Cancer, Volume 14, March 2014, 173-185)

4.1.6 RET Point Mutation in Sporadic MTC (SMTC):

Approximately 50-60% of specimens from patients with SMTC, are reported to contain somatic but not germline RET mutations *(407)*. The most common somatic mutations occur in exons 14, 15 and 16 while less common somatic mutations in exons 10 and 11 are found associated with poor prognosis *(409)*. Murra et al, have demonstrated somatic mutations in 64.7% of MTC tumours. Exon 16 was the most frequently affected (60.6%), followed by exon 15, while exons 5, 8, 10-14, were less affected. Mutations in exons 15 and 16 were associated with higher prevalence of persistent, multifocal MTC with a spread in regional lymph nodes, while mutations in exons 5, 8, 10-14, were associated with the most indolent course of MTC. Single nucleotide polymorphism (SNP) of RET has been shown to be more

frequent in patients with SMTC compared with healthy subjects, associated with an earlier age of MTC, and with higher calcitonin levels. The real significance of the observed mutations is not clear. At present, genetic testing for tumour mutations of the RET is not part of the routine practice in patients with SMTC, as the clinical utility is still undefined *(79, 410, 411)*. However, screening of individuals with apparently sporadic MTC may uncover germline RET mutations in approximately 7% of cases *(411)*. Thus, it might be useful to perform RET genetic testing in all patients with MTC, even in those with apparently SMTC.

4.2 Aims and Objectives:

Much prospective data have been obtained since the discovery of RET mutations, but the true frequency of inherited disease among apparent sporadic MTC cases and the frequency of *de novo* germline mutations of the RET proto-oncogene remain a matter of discussion. The extent of genetic analysis needed for exclusion of inherited disease is also not well defined. Although, every mutations do not need to be included in routine diagnosis, there have only been a few attempts to define the risk of inherited disease in relation to the range of genetic investigations. Published data indicate differences among the frequencies of mutations in exon 10, 11, 13, 14 and 16 mutations in analyzed populations.

In the present pilot scale analysis with only 25 samples (which is a part of ongoing larger analysis in collaboration with Tata Cancer Hospital, Mumbai) our goal was to find the prevalence of reported and novel mutation and estimate the risk associated with these mutations in MTC patients (including both sporadic and hereditary cases) in the Indian population.

4.3 Materials and Methods:

4.3.1 Acquisition of Tumour Tissue Specimens:

Frozen and paraffin embedded thyroid tissues from thyroid cancer patients who had attended the clinic between 2000 and 2010 were retrieved from the Tissue Repository of Tata Memorial Hospital, Mumbai, India for analysis of RET gene mutations at exon 10, 11, 13, 14 and 16. A total of 25 specimens with adequate clinical and pathological information were studied. Patients were staged using the tumour-node-metastases (TNM) system and classified according to the presence of extra-thyroidal extension, cervical nodes and distant metastases.

4.3.2 Molecular Analysis:

4.3.2.1 Genomic DNA Extraction:

Nucleic acid extraction was performed as described previously in the Chapter 2, section 2.1.

4.3.2.2 Detection of Point Mutations:

PCR Amplification of RET gene exons (Exon 10, Exon 11, Exon 13, Exon 14, Exon 16) was performed using the DNA extracted from the tumour samples.

Amplifications by PCR were carried out using one set of forward and reverse primers specific for each RET gene exon. The oligonucleotide primer sequences (Xcelris Labs Ltd, India) are presented in Table 4.1. PCR was performed with 100ng of genomic DNA, using the following conditions specific for each primer set, for 35 cycles of amplification. Reactions contained final concentrations of 20 mm Tris-HCl (pH 8.4), 50 mM KCl, 400um dNTPs (Invitrogen), 1.5 mm MgCl2, 10 pmol of both primers, and 1.5 IU of Taq DNA polymerase (Invitrogen). Negative controls for PCRs, in which the template was replaced by sterile water, were included in each experiment. DNA integrity and efficiency of PCR were confirmed in each sample by PCR for the housekeeping gene GAPDH. PCR products were

analyzed by electrophoresis in a 5% polyacrylamide gel and purified by running in 1.5% Agarose gel.

PCR amplified products were gel purified using HiPurA ^{TM} Gel Purification Kit following manufacturer's instructions. Sequencing of gel extracted amplicons was performed using Big Dye Terminator Kit as described in Chapter 2.3.

Table 4.1: Primers for amplification of RET Exons 10, 11, 13, 14 and 16

primers	Sequence 5'-3'	T_m	Amplicon
RET 10F	GGAGGCTGAGTGGGCTACG	63.2	210 bp
RET 10R	GTGCTGTTGAGACCTCTGTGG		
RET 11F	CCTCTGCGGTGCCAAGCCTC	63.1	234bp
RET 11R	CACCGGAAGAGGAGTAGCTG		
RET 13F	GCAGGCCTCTCTGTCTGAACTT	64	295bp
RET 13R	GGAGAACAGGGCTGTATGGA		
RET 14F	TCCTGGAAGACCCAAGCTGC	65	300bp
RET 14R	CTGGGTGCAGAGCCATATGC		
RET 16F	AGGGATAGGGCCTGGGCTTCT	63.1	194bp
RET16R	TAGGTGATTTTGGTCTAGCTACAGT		

4.3 Results:

4.3.1 Prevalence of RET point Mutations:

Amplicons of different RET gene exons from genomic DNA from the samples on 5% polyacrylamide gel electrophoresis, indicating specific amplification of the exons is shown in Fig. 4.3. The nucleotide sequences of the exons identified the PCR products as RET exons 10, 11, 13, 14 and 16, with complete homology to the exons, with exception of the mutated nucleotides. The representative sequencing electropherogram of all the exons demonstrating the normal sequences and the mutation observed is given in figure 4.4 and 4.5.

From our preliminary studies, RET point mutations were observed in 14 (56%) out of 25 patients, indicated in Table 4.2. Mutation in RET exon 13 was having the highest prevalence being found in 12 (48%) patients. RET exon 10 and 11 were having mutations in 4 (16%) and 1 (4%) patient respectively. We did not find any mutation in exon 16 (Fig 4.6).

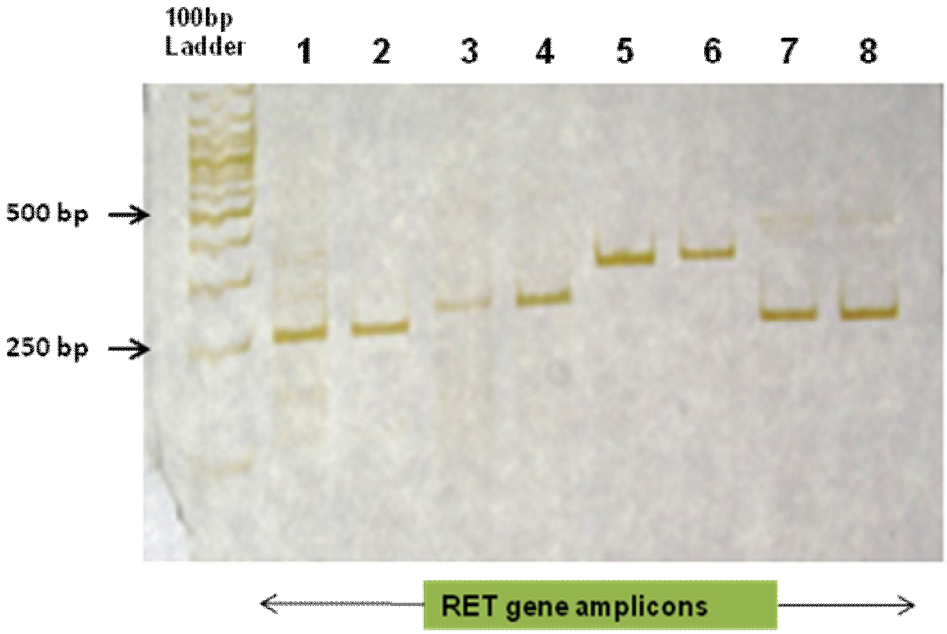


Fig 4.3: Amplicons of different RET gene exons: 100 bp DNA ladder followed by Exon 10 (lane 1,2); Exon 11 (lane 3, 4); Exon 13 (lane 5); Exon 14 (lane 6); Exon 16 (lane 7, 8)

Table 4.2: The results of RET (Exon 10, 11, 13, 14, 16) point mutation of each patient along with their pathological attributes

RET exons	Total (%)	No of Patients			
		Positive		Negative	
		N	%	n	%
RET exon 10	56	4	16	21	84
RET exon 11		1	4	24	96
RET exon 13		12	48	13	52
RET exon 14		1	4	24	96
RET exon 16		0	-	25	-

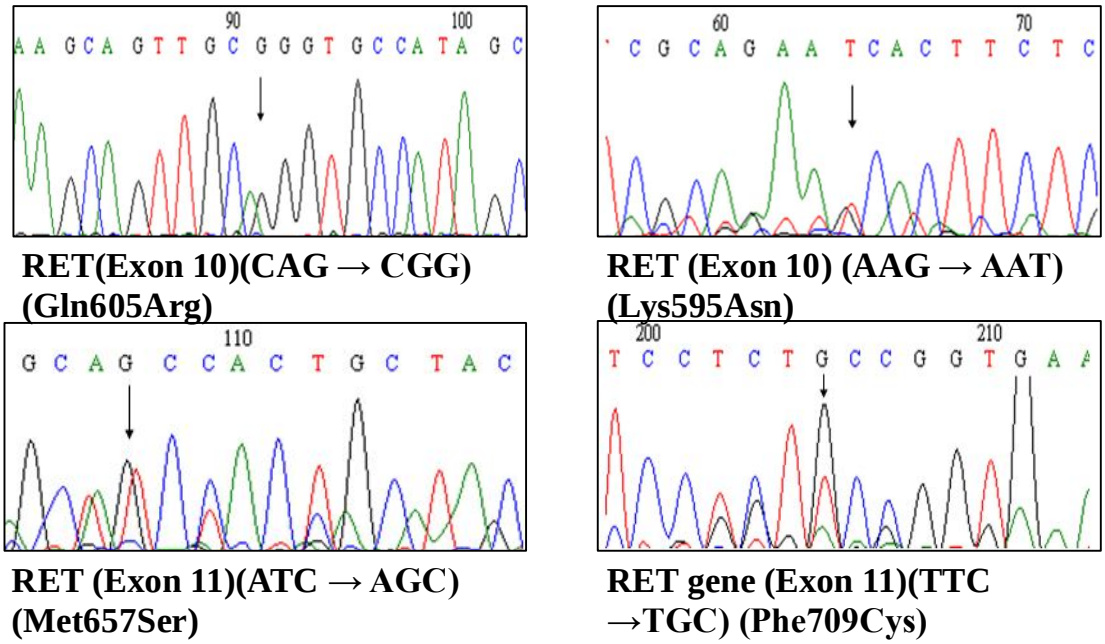
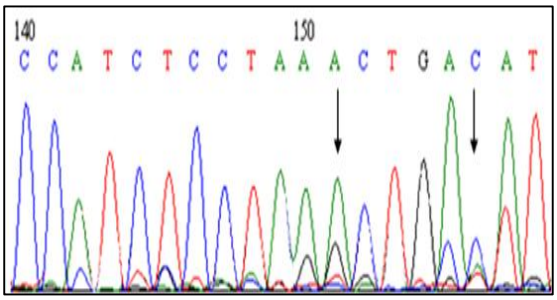
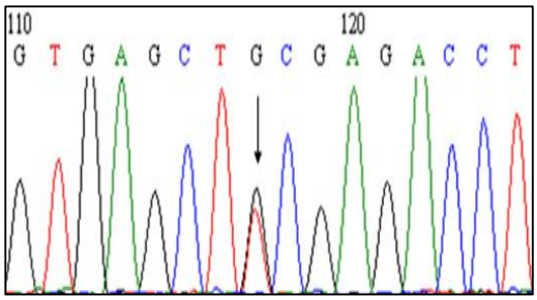


Fig 4.4: Sequencing electropherogram of RET gene, exon 10 and exon 11. A. Codon 595 and 605 showing amino acid change of Gln to Arg and Lys to Asn respectively. B. Codon 656 and 709 demonstrating amino acid change of Met to Ser and Phe to Cys respectively

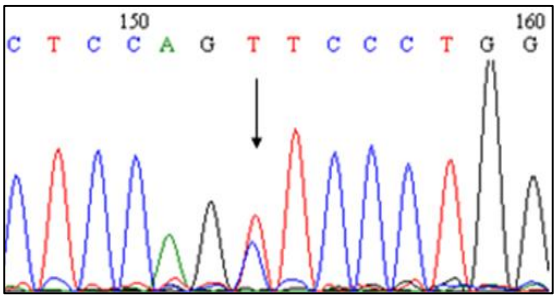
Sequence analysis of the critical RET gene exons 10, 11, 13, 14 and 16 were compared with reference RET gene nucleotide sequence. For both DNA (NC_000010.11) and mRNA (NM_020975.4) were used for sequence alignment and identification of the SNPs. We observed transversion mutation in exon 10 codon 595 (AAG to AAT) with amino acid change of Lys to Asn. Another transition mutation in codon 605 (CAG to CGG) resulting into replacement of Gln with Arg. Exon 11 harboured a number of rare SNPs in codon 657, codon666, codon 672, codon 673, codon 679, codon686 and codon 709. Among these nucleotide alterations codon 676 and codon 709 resulted into Phe to Ile and Phe to Cys conversion. Genetic variants at codon 769 observed in exon 13 was a polymorphism with nucleotide CTT to CTG conversion resulting into alternate codn for Leu. Similar polymorphism was observed for exon 14at codon 836 with nucleotide change producing alternate codon for Ser.



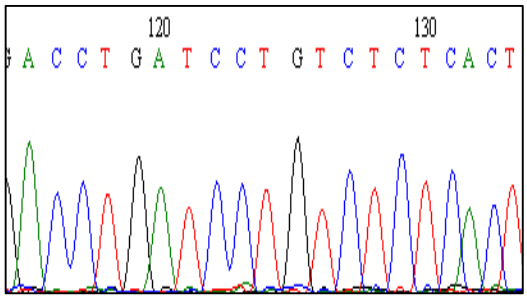
RET (Exon 11) (GCT → ACT) (Ala672Thr)
RET (Exon 11) (GAG → GAC) (Glu673Asp)



RET (Exon 13)(CTT → CTG) (Leu769Leu)



RET gene (Exon 14) (AGC → AGT) (Ser836Ser)



Stretch of Sequence in Benign Tissue

Fig4.5: Sequencing electropherogram of RET gene, exon 11, 13 and 14 . A. Codon 672 and 605 showing amino acid change of Ala toThr and Glu toAsp respectively. B. Codon 769 and 836 of exon 13 and 14 shows polymorphic changes to same amino acids.

4.3.2 Clinico-pathological correlation:

Though this analysis, to date, contains few samples for precise interpretation, an attempt has been made to deduce correlation with clinical parameters using Fisher's Exact Test and Multivariate Logistic regression analysis.

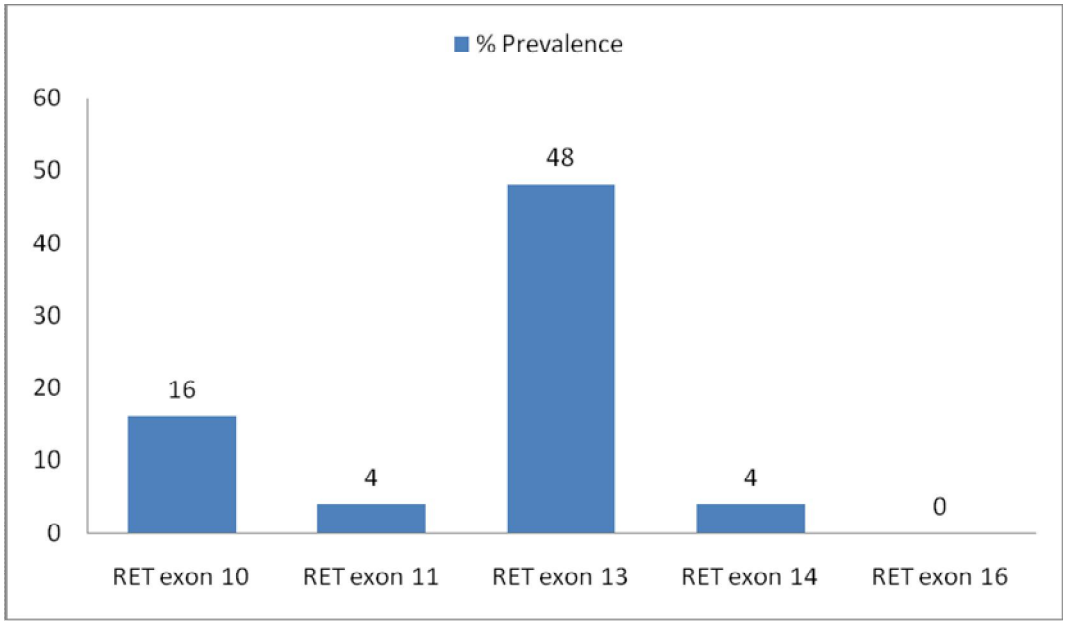


Fig:4.6: Frequency of genetic alerations in different exons of RET proto-oncogene

The mean age ± SD was 43.5 ± 10.0 yr, and the mean tumour size ± SD was 2.6 ± 1.5 cm. The association between RET point mutation and various clinico-pathological parameters were assessed in 25 samples (Table 4.3). The RET mutation was significantly associated with the presence of metastasis (Fisher's test, $P < 0.05$ and Univariate regression, $p<0.005$). RET point mutation was also associated with tumour stage ($p<0.05$). No significant association was found between the presence of RET mutation and gender of patients ($p=1$) ie. there is exactly equal probability of occurrence of this rearrangement in both the genders. Age ($p=0.41$), Tumour size ($p=0.45$) and extra-thyroidal extension ($p=0.12$) also had no association.

Although Fisher's exact test showed association with two different clinico-pathological parameters, Multivariate regression analysis revealed no significant association at 95% confidence interval.

Table 4.3: Association between *RTE* mutation and clinico-pathological parameters in medullary thyroid carcinoma

	RET Mutation				Fisher's test (2 tail)	Univariate Regression
	Positive		Negative		*P*	*P*
	N	%	N	%		
Tumour Stage					0.017*	0.004
T I	4	36	7	64		
T II	1	25	3	75		
T IIIIV	8	88.9	1	11.1		
Gender					1.0	0.69
Male	10	58.8	7	41.2		
Female	4	50	4	50		
Age					0.41	0.58
≥45yr	4	40	6	60		
<45yr	8	61.5	5	38.5		
Metastasis					0.013*	0.004
Yes	8	88.9	1	11.1		
No	5	33.3	10	66.6		
Extra-thyroidal Extension					0.12	0.071
Yes	8	72.7	3	27.2		
No	5	61.5	8	38.4		
Tumour size (mm)					0.45	0.19
range, mean ± SD	1	33.3	2	66.6		
<10 mm	8	50	8	50		
10–40 mm	4	80	1	20		
>40 mm						

* $p<0.05$

Table 4.4: Summary of Point Mutations Detected in the RET Proto-Oncogene in MTC

Exon	Codon	Nucleotide Change	AminoAcid Change
10	595	AAG to AAT	Lys to Asn
10	605	CAG to CGG	Gln to Arg
13	769	CTT to CTG	Leu to Leu
14	836	AGC to AGT	Ser to Ser
11	657	ATC to AGC	Met to Ser
11	666	AAG to AAC	Lys to Asn
11	672	GCT to ACT	Ala to Thr
11	673	GAG to GAC	Glu to Asp
11	676	TTC to ATC	Phe to Ile
11	686	AGC to ACC	Ser to Thr
11	709	TTC to TGC	Phe to Cys

4.5 Discussion:

The confirmation of RET gene mutations as prognosis of clinically diagnosed MTC is gaining importance. In addition, screening of first degree relatives for the mutations indicative of high risk of neoplasias in the members of family mandates analysis of the mutations in MTC. The RET gene mutations are a critical factor in patient management, e. g. to decide on total or partial thyroidectomy or the age at which the child should be subject to surgery *(412, 413).*

Vast amount of data has been reported to unequivocally establish the value of RET gene mutations in prognosis and management of inherited and sporadic MTC patients, as also to

predict risk for family members *(407, 410, 414)*. However, there is paucity of data on RET gene mutations in patients from our Indian population.

We have initiated the investigation on RET gene mutations in MTC patients and observed genetic aleration in RET gene in 56% (14/25) clinically diagnosed MTC patients. Investigations of patients MEN (2A/2B) status and presence of mutation in family members will provide more understanding about our population *(415, 416)*.

From the present pilot analysis, the most frequently observed mutation was in exon 13 at codon 769 (48% patients), followed by mutations in exon 10 (16%), exon 11 (4%) and exon 14 (4%). Among all the genetic alterations observed CAG →CGG causes non-polar amino acid Gln to polar amino acid Arg in the translated protein. This may have effect on constitutive activation of RET tyrosine kinase domain. Another activating mutation that altered the polarity of the amino acid includes Lys to Asn (AAG→AAC at codon 666) in exon 11. Mutation in the same exon at codon 676 (TTC→ATC) and codon 709(TTC→TGC) caused conversion of hydrophobic amino acid Phe to nonpolar Ile and Cys respectively. These novel missense mutations observed in following codon: (Lys595Asn), (Glu605Arg), (Met657Ser), (Lys666Asn), (Ala672Thr), (Glu673Asp), (Phe676Ile), (Ser686Thr) and (phe709Cys) ,which code for cysteine rich domain ,transmembrane domain and tyrosine kinase domain, may have role in upregulation of downstream signaling pathways *(417, 418)*.

The most frequent alteration at codon 769 (CTT to CTG) observed in 13 (52%) patients appear to be a polymorphism with no change in amino acid sequence (Leu→Leu). Another polymorphism was found at codon 836 (AGC→AGT) in one patient. But we could not find major mutations at codon 609 and 634, reported earlier in familial MTCs and MEN2A/B patient on various European sample groups. Though defined as polymorphisms, it cannot be ruled out that these alterations, observed in patients, could have a low level of influence, acting as genetic modifiers and associated with a small increased relative risk for

development of the disease. However the statistical analysis performed included both polymorphism and mutations, due to smaller sample pool, can prove erroneous and not reflect the true effect of RET on prognosis of MTC *(419-421)*.

A study with a larger number of patient samples and controls will be needed to confirm the same.

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Conclusion

There is an increase in the incidence of thyroid cancer mainly due to awareness among the public and general practitioners and early detection using sensitive methods, like, ultrasound, radioiodine scans and fine needle aspiration cytology. There is statistical evidence to show that thyroid cancer is more frequent in women. Given the relative success we have had in its treatment, there is a need to use the information gathered during diagnosis and follow-up by better understanding the factors that underpin this malignancy and exploring better strategies for diagnosis, treatment, and follow-up. To do so, we must take full advantage of the tremendous advances that have been made in molecular biology, biotechnology and bioinformatics.

Early stage thyroid cancer follows standard treatment modality and often cured by surgical resection and ablation of remnant thyroid tissue with radio-iodine (I-131). However, advanced stage disease is still incurable, and current treatment with chemotherapy has not been shown to significantly improve morbidity or mortality. To address this problem we started by screening BRAF; which is one of the major isoform of MAP Kinase pathway gene RAF. Since the initial discovery of *BRAF* mutation in human cancers, there have been more than 40 mutations identified in the *BRAF* gene in different cancers. Amongst these mutations the T1799A point mutation was and still is the most common and accounts for more than 90% of all the mutations found in the *BRAF* gene. We for the first time demonstrated that the frequency of occurrence of *BRAF* mutation in sporadic PTCs, mainly in the classic variant of PTC is higher in our patients and confirmed that this mutation is strongly associated with the papillary growth pattern. Among 140 patients who registered in Tata Memorial Hospital, Mumbai, from different parts of the country, $BRAF^{V600E}$ mutation was found in 46 out of 86 PTC patients (53.4%), in comparison with observations from other Asian countries, Japan 36 %, Taiwan 46.6 % and Ukraine 22.9 %. Also, the poorly differentiated carcinoma (PDTC) and tall cell variants, which were more aggressive in nature, harboured *BRAF* mutation more frequently as expected. Further, we found significant correlation of *BRAF* mutation with nodal metastasis ($p < 0.005$), extra thyroidal invasion ($p < 0.005$) and tumour stage ($p < 0.005$). Thus, our study also disproved the controversy on antagonistic role of BRAF mutation in tumour progression. We also understood from

multivariate regression analysis that, *BRAF* mutation should not be considered independently but should be reviewed along with tumor stage, as another prognostic factor, to determine risk within a particular subtype of PTC. Thus our finding suggested that morphologic typing in combination with *BRAF* mutation status appears to be better prognostic indicator in PTC.

In Mumbai, 3-4% of the cancer patients are reported to suffer from thyroid neoplasia. To the best of our knowledge ours is the one and only published data on the prevalence of *BRAF* mutation in thyroid cancer in patients from the Indian sub continent. Relevantly, considering that the prevalence is >50% in this cohort, *BRAF* gene can be a promising target for small molecular inhibitors for better prognosis of radioiodine refractory thyroid carcinoma patients.

In the second part of this chapter we tried to evaluate a special case of aggressive Tall cell variant with multiple recurrences. A concordance of BRAF mutation with expression of Muc1 and other immune-histochemical prognostic markers were found in this case. We also attempted to establish a link between molecular diagnosis and conventional scanning methods (^{131}I Scintigraphy and ^{18}F FDG PET). In this patient, all the metastatic tissue specimens collected at different times as the disease progressed, showed $BRAF^{V600E}$ mutation, indicating that persistent presence of constitutively expressing BRAF may have a role in multiple recurrence.

Some recent literatures also revealed that the aggressiveness of the disease in terms of clinical behavior could be related with the genetic mutation that is associated with aggressive features in Differentiated Thyroid CA. Investigation in more patients with a similar history is in progress in our lab and if proven would open up further research with BRAF specific therapies (e.g. BAY 43-9006, AMG 706, Vemurafenib) in this group of patients.

Activating *H, K,* and *N RAS* mutations, a most common type of abnormality of a dominant oncogene in human cancer have been addressed in the third part of this chapter. Several prospective and retrospective analyses in thyroid cancer showed that *RAS* mutations are associated with poor prognosis with a mush varying frequency ranging from 7% to 62%. Further, their prevalence in specific thyroid histotypes was also unclear, necessitating to

investigate the true frequencies in our own population. Using Competitive Allele Specific PCR method (KASP), a technique with much lower turnaround time, we demonstrated that different isoforms of *RAS* mutations define a specific subset of thyroid carcinoma. This is indicated by the close association between oncogenic *NRAS* C13GC with PTC, HRS C13GD with PDTC and exclusive presence of *KRAS* C12GD with PDTC and ATC. Relevantly, we found relative higher prevalence of *RAS* mutations in PDTC of FTC origin which supports the role of *HRAS* and *KRAS* in dedifferentiation of well differentiated follicular thyroid carcinoma. Moreover, analysis of *KRAS* codon 13 and 61 may reveal some more intriguing role in disease progression.

In summary, our results predict that *NRAS* mutations may be a potential prognostic marker for aggressive behavior and poor outcome of thyroid cancer. Additional investigations of activating mutations of *KRAS* (codon13, 61) and *HRAS* (codon 12) may reveal some more important role of these RAS isoforms in disease progression and help in developing for novel treatment modalities.

We have also moved a step towards understanding the role of chromosomal rearrangements in thyroid neoplasia. *PAX8/PPARγ* fusion oncogenes, one of the most recently explored determinants of thyroid malignancy, was investigated in the third chapter. Our results raise some interesting issues on the origin and putative relationship between FVPTC and FTC. In a study group of 62 patients, *PAX8/PPARγ* fusion oncogene was detected in ~21% cases with much higher prevalence in FVPTC (33%). Detailed histopathological observation of cell nucleus under a microscope reveals a pattern that, this fusion oncogene may be involved in follicular growth pattern of PTC but need additional molecular alterations for the onset of malignancy. Our results are similar to that reported by scientists for other ethnic communities. The observation that this fusion gene confers higher invasive potential to the neoplastic cells in tumour microenvironment is a significant one. Relevantly, no BRAF

mutations in the V600E hot-spot coexisted with rearrangement in any of the follicular tumours. Taking into consideration the mutual exclusive prevalence of *BRAF* and *PAX8/PPARγ* in FVPTC and based on genome hybridization data from literature, we predicted that PTC, FVPTC and FTC can be considered as different subgroups where FVPTC shares a commonality with rest of the two histotypes in several aspects.

Analysis of another chromosomal rearrangement, leading to the generation of chimeric *RET/PTC* oncogenes, has been described in later part of the third Chapter. There is considerable variation in prevalence of *RET/PTC* rearrangements in published reports from different ethnicities. Though its occurrence is lesser than that of *BRAF* mutations, there is a controversy on its role in thyroid tumourigenesis and coexistence with *BRAF*. In this study group of 62 samples, significant prevalence of *RET/PTC* 1 and 2 (43.7%) was found in PTC histotype with coexistence of *BRAF* in 28% cases. Exclusive presence of *RET/PTC* in 13% cases of PTC envisage the possibility of combining *BRAF* with *RET/PTC* analysis to increase the fraction of identifiable PTC. On the other hand, a low prevalence of *RET/PTC* (16%) and *BRAF* (11.7%) in FVPTC urges the need for novel molecular markers like *PAX8/PPARγ* (33.3%) for specific FVPTC subtype that can be used in adjunct to *RET/PTC* and *BRAF* detection.

Our final chapter deals with RET gene mutations in the onset of C cell derived medullary thyroid carcinoma. The RET gene mutations status is gaining importance in patient management in the developed countries. Screening of first degree relatives of MTC patients have become a deciding factor for total or partial thyroidectomy or the age at which the child should be subject to screening for MTC.

In spite of vast data available from literature worldwide on possible oncogene mutations, there is paucity of data on RET gene mutations in patients from India. Our preliminary investigation has revealed 56% prevalence of altered RET oncogene in clinically diagnosed MTC patients with codon 769 at exon 13 as most prevalent hotspot. A larger and prospective investigation of patients with

MEN (2A/2B) and presence of mutation in family members will be necessary to calculate the diagnostic and prognostic utility of RET gene alterations in our population.

To conclude, the existing clinical management of patients with thyroid nodules classified as indeterminate is a huge social cost for both patients and the health system. The development of a reliable genetic test prior to surgery that can distinguish between benign and malignant thyroid nodules and overcome the pitfalls of an undiagnosed nodule is mandatory. An increasing number of publications have described new potential molecular markers for diagnosis of thyroid nodules.

Through this work, an effort has been made to explore the potential of several genes as diagnostic and prognostic markers in thyroid carcinoma. However, it is seen that a lot more is required to be done. From the initiation of this project to the present day, discoveries and inventions in different fields of study in the context of cancer have led to tremendous advancements in biologic techniques. The use of karyotypic analysis, fluorescent in situ hybridization, candidate gene sequencing, microarray expression analysis, and next generation sequencing, whole-genome association and molecular imaging are presently assisting in determining the molecular defects causing thyroid cancer. The stronger correlation of disease phenotype with genomics, transcriptomics, proteomics and metabolomics of the cancer cell will provide us with clear classifications and will allow us to develop guided management strategies to optimize the time of intervention and schedule follow-up and manage targeted therapy. As our understanding of the depth of these correlations increases, we can look forward to better refining our treatment and management regimes in order to improve the quality of life and provide better care to the thyroid cancer patients in our country.

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