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PROGNOSTIC MARKERS IN THYROID NEOPLASIA

A thesis submitted for the degree of
Doctor of Philosophy

by

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Trinity College
University of Dublin

Michelmas Term
1999

Supervisor:
Prof Eamon C Sweeney, MA, MD(NUI), FRCPI, FRCPath, FTCD.

DECLARATION

I declare that this thesis is my own work, and has not been submitted previously for a PhD degree at this or any other university. I agree that the library may lend or copy this thesis on request.

Orla Sheils

For my parents

Etta and in memory Edward.

Beware lest you lose the substance by grasping at the shadow

- Aesop.

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To begin with, I would like to thank my supervisor Prof. Eamon Sweeney for all his help and encouragement. His ability to think laterally when presented with unexpected results and his keen sense of grammatical accuracy have been an education in themselves!

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And finally, thanks and apologies to Fritz – who has gone without more walkies than any dog should have to over the last months. Get your lead – We're off.

LIST OF ABBREVIATIONS

A	adenosine
A_{260}	absorbance @ 260nm
A_{280}	absorbance @ 280nm
AMV	avian myeloblastosis syndrome
APES	3-aminopropyltriethoxy silane
bp	base pair
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BD	Big Dye™
BSA	Bovine Serum Albumin
C	cytosine
C_T	threshold cycle
CCD	charge-coupled device
cDNA	complementary DNA/ copy DNA
DAB	3',3'-diaminobenzidine
DAPI	diaminophenylindole
DEPC	diethylpyrocarbonate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
ddNTP	di-deoxynucleoside triphosphate
DMF	dimethylformamide
EDTA	ethylenediaminetetra-aceticacid
EtBr	ethidium bromide
FAM	6-carboxy fluoroscein
FFPE	formalin fixed paraffin embedded tissue
G	guanine
GAPDH	glyceraldehyde phosphate dehydrogenase
H_2O_2	hydrogen peroxide
H&E	haematoxylin and eosin
IPTG	Isopropyl-Thio-β-D-Galactopyranoside

LB	Luria-Bertani
$MgCl_2$	magnesium chloride
MMLV	Moloney murine leukaemia virus
mRNA	messanger RNA
MuLv	murine leukaemia virus
NaCl	sodium chloride
°C	degrees Celcius
OD	optical density
ODN	oligonucleotides
POP4™	performance optimised polymer 4
POP6™	performance optimised polymer 6
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RT	reverse transcription
T	thymidine
T_m	melting temperature
T_a	annealing temperature
TaqMan	TaqMan PCR
TAE	Tris – acetic acid – EDTA buffer
TAMRA	6-carboxy-tetramethyl-rhodamine
TBS	tris buffered saline
Tris	Tris(hydroxymethyl)methylamine
TSR	template suppression reagent
X-Gal	5-bromo-4-chloro-3-Indolyl-β-D-Galactopyranoside (β-Gal Substrate)

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SUMMARY

Prognosis in thyroid carcinoma is usually assessed on the basis of criteria, which include patient age and histological type, grade and stage of tumour. It is well recognised, however, that while occasional tumours with adverse histological features, such as necrosis or high mitotic rate, are cured, other seemingly well-differentiated ones may progress to a fatal outcome.

The purpose of this project is to evaluate a panel of potential prognostic markers in a series of thyroid neoplasms. The assessment began using a combination of tinctorial and immunohistochemical techniques to assess cell kinetics, oncogene expression (p53) and synthetic function, and progressed towards a more molecular approach employing TaqMan™ quantitative RT-PCR to examine TSHr expression and *ret*/PTC-1 activation.

The results obtained from immunohistochemical evaluation of the sample cohort showed a greater proliferative activity among ATC, compared with other forms of thyroid neoplasia. This finding was matched by greater p53 and EGFr expression, and a correspondingly higher apoptotic rate. Relative TSH receptor (TSHr) expression as detected by RT-PCR was markedly decreased in undifferentiated tumours.

Quantitation of TSH receptor (TSHr) expression was performed using TaqMan RT-PCR. A TSHr index was devised to normalise the expression data for variations in extent of degradation due to different fixation, processing or storage conditions. The data obtained using this assay enabled the categorisation of tumours into well- and poorly-differentiated sub-types.

Activation of the *ret* proto-oncogene has been described among papillary thyroid carcinomas (PTC). The most commonly detected variant of *ret* activation is *ret*/PTC-1, accounting for approximately 70% of *ret* activated PTC and involves a translocation between c-*ret* and another gene termed H4. H4 encodes the

synthesis of a putative cytoskeletal protein. TaqMan based RT-PCR assays were designed for analysis of *ret*/PTC-1 and H4.

ret/PTC-1 activation was not detected in the FTC or FA groups, while H4 was detected in all these cases. Thirty three percent of all cases (PTC +ATC) were found to express the chimeric RNA characteristic of *ret*/PTC-1 with 87.5% of ATC positive. Those cases which expressed activated RET failed to express H4.

Another important observation in the assessment of *ret*/PTC-1 activation among PTC, was the incidence of chronic lymphocytic thyroiditis in association with *ret*/PTC-1 positive tumours. This gave rise to the speculation that *ret*/PTC-1 may influence the growth pattern in PTC, and may also be involved in the initiation of an immunological response similar to that seen in thyroiditis.

Examination of a series of non-neoplastic thyroid tissues for *ret*/PTC-1 activation was performed. The data obtained demonstrated that >95% of Hashimoto thyroiditis tissues examined had activated RET expression. In the majority of cases, this phenomenon occurred with no evidence of associated PTC. The conclusion from this finding was that Hashimoto thyroiditis might represent an early malignant or pre-malignant state, which is detected by the immune system of a subset of the population

ret/PTC-1 transcripts within the cell were demonstrated using a novel in-situ TaqMan assay. This technique confirmed the transcript to be cytoplasmic. Further, where cells clustered together in follicle formation the staining pattern was discrete, and formed a distinct border along the luminal edge of cells. This data reinforces the concept that *ret*/PTC1 is involved in morphogenesis in PTC.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Historical interest in the Thyroid

The thyroid is the largest discrete endocrine organ, weighing 20-25g in a normal adult. It is a purple-brown gland consisting of two lateral lobes linked by an isthmus that lies anterior to the second and third tracheal rings. The thyroid is composed of follicles ranging in size from 50 to 900nm in diameter, which consist of a single layer of cuboidal or columnar epithelial cells within a basement membrane.

Goitre and mental retardation, the effects of impaired thyroid hormone production, are endemic in many parts of the world where there are inadequate amounts of iodine in the diet. The term goitre indicates enlargement of the thyroid gland (from the Latin Guttur, meaning throat). The earliest documented descriptions of goitre were in China in 2700 BC.

Galen, who thought its function was to lubricate the pharynx initially described the thyroid in the second century AD, but it was the Italians of the Renaissance period who first recognised the normal thyroid gland. Leonardo da Vinci (1452-1519) and Andreas Vesalius (1514-1564) described it as two glands and Bartholemus Eustachius (1520-1574) described a single '*glandulam thyroideam*' (from the Latin meaning shield-shaped). In 1619 Fabricius ab Aquapendente (1534-1634) recognised that goitres arose from the thyroid, and in 1776 Albrecht von Haller (1708-1777) of Berne classed it as a ductless gland. Autoimmunity to organ specific antigens in the thyroid is another major factor in the pathogenesis of several thyroid diseases. It is of historical interest that hypothyroidism was the first endocrine deficiency to be successfully treated, the missing hormone being replaced by subcutaneous injection of 'thyroid juice'.

Cancer of the thyroid was a disease recognised in the 18th Century, and John Athenbury (1764-1831) of London described one variety then named 'medullary sarcoma' as a 'terrible affliction'. Two types of lesion with better prognoses were also recognised. These were the so called '*papilliferous* (papillary) and

encapsulated' lesions. The pathological classification of tumours of the thyroid follicular cell was very confused for many years, but in 1925 two main types of lesion were recognised; malignant 'adenomas', which metastasised early via the blood stream, and papillary adenocarcinomas, which were less malignant and spread mainly by the lymphatics. From then on pathological classification and surgical practice advanced together. By the 1950's, cancer was being diagnosed earlier than ever. Papillary growths could often be removed by total lobectomy, with excision of local lymph nodes. The adenocarcinomas were treated by extended thyroidectomy and radioiodine therapy.

Since the 1970s fine needle aspiration biopsy cytology has greatly contributed to the early diagnosis of cancer, especially in those patients with solitary nodules.
Thyroid carcinoma, although representing no more than 1.5% of all cancer deaths, remains the most common malignancy of the endocrine system. Differentiated thyroid carcinoma is the term used to describe those tumours arising from follicular and parafollicular cells which can be distinguished by their histological pattern. The term encompasses follicular (FTC), papillary (PTC) and medullary (MTC) carcinomas, and all their histological variants. In general these tumours follow a predictable clinical course depending on their histological subtype, though it has long been recognised that a proportion of tumours in any given category will behave in a fashion which is not the norm. It was the identification of aberrant behaviour that led, in the seventies, to the reclassification of many cases of 'follicular carcinoma' as follicular variants of PTC, with a significantly different prognostic outlook. Similarly, several other variants within histological subtypes, all with differing prognoses have since been identified.

It has been suggested that follicular tumours represent an adenoma-carcinoma sequence, analogous to that found in the colon, and when malignant transformation occurs, metastasise widely via the bloodstream. The majority of thyroid tumours however are papillary carcinomas. These appear to arise directly as carcinomas, which classically evolve slowly over a number of decades. Those that progress, metastasise locally via lymphatics and generally have a better

outcome than their follicular counterparts. However, there are exceptions and reasons for these exceptions are not clear. Both types of tumour may undergo de-differentiation and give rise to the much more aggressive anaplastic carcinoma. Generally the prognosis in thyroid carcinoma is dependent on the patient's age and stage of tumour at time of diagnosis. However, tumours with several adverse features such as necrosis, or a high mitotic index may follow an indolent course while other seemingly inert ones may rapidly progress and have a fatal outcome.

Recent advances in molecular biology have improved our understanding of the pathogenesis of thyroid carcinomas. Re-arrangements of the tyrosine kinase domains of RET and TRK genes with the amino-terminal of an unlinked gene are found in some papillary carcinomas. RET re-arrangements are found in 3-33% of PTCs unassociated with irradiation, and in 60-80% of radiation induced PTC. The frequency of TRK re-arrangements is much lower. Activating point mutations of the *ras* genes are found with a similarly high frequency in thyroid adenomas and follicular carcinomas as re-arrangements of *ret* in PTC. Inactivating point mutations of the p53 tumour suppressor gene are rare in patients with differentiated carcinomas of the thyroid but are common in anaplastic variants. Hence, it has been suggested that two oncogenes may have transforming effects on one cell type but induce dramatically different phenotypes.

The overall aim in cancer research is to understand the etiologic basis for carcinogenesis, with the long-term objective of reducing cancer incidence. Epidemiological studies incriminate environmental factors as well as genetic predisposition in the aetiology of cancer. The recognition that cancer is a multi-step process and the rapid elucidation of these steps may facilitate targeting of therapy. In histological practice, cases of thyroid carcinoma, which do not behave true to form are encountered with depressing regularity. The aim of this project is to yield information, which will allow more precise grading and prognostication within the different categories of thyroid carcinoma.

1.2 The origin of the thyroid gland

The thyroid gland appears by the end of week three of gestation as an endodermal thickening on the ventral surface of the pharynx. Descent of the thyroid gland from its origin at the foramen cecum can be attributed to elongation of the embryo and anterior growth of the tongue. The developing thyroid remains connected to the foramen cecum by a thyroid diverticulum and subsequently by a thyroglossal duct comprising a hollow cord of cells that becomes solid by the sixth week of life as the cells migrate caudally. At the end of week five, the duct loses its lumen before it fragments. The advancing end becomes bi-lobed and differentiation into thyroid parenchymal cells commences.

Epithelial cell proliferation accounts for initial thyroid growth until two months gestation. By the end of the third month the epithelial plates have been completely replaced by primary follicles. Subsequent thyroid growth is attributed to budding and division of these primary follicles and after the fourth month to increase in follicle size. Colloid formation within the follicles begins at eleven weeks. This corresponds to the time at which radioactive iodine uptake by the developing thyroid can be detected.

The thyroid follicular cell is highly differentiated and has evolved an efficient iodine concentrating system (figure 1.1). Accumulated iodine is employed for the synthesis and storage of thyroid hormones. Thyroid cells are programmed to aggregate into follicles with a central space for the storage of thyroglobulin. This organisation necessitates the polarisation of the thyroid cell. The basal pole is involved in the importation of iodine, which is then transported to the apex where it is used to iodinate thyroglobulin under the enzymatic action of peroxidase.

TSH released by the pituitary gland predominantly regulates thyroid cell growth and secretion. It activates both the adenylate cyclase and phospholipase C pathways. The activation of adenylate cyclase and cyclic adenosine monophosphate (cAMP) accumulation results in dissociation of the regulatory and

catalytic subunits of cAMP-dependent protein kinase-A, with subsequent phosphorylation of various cellular proteins. Activation of phospholipase C causes hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP2) with formation of diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). DAG activates protein kinase C and IP3 increases intracellular calcium concentrations. The concentration of TSH necessary to activate phospholipase C is greater and its action slower than that necessary for adenylate cyclase activation. cAMP induces DAG synthetase in the thyroid thus providing substrate for protein kinase C, allowing activation of this pathway at physiologic TSH concentrations.

1.3 Physiology of the thyroid gland

The basic functional unit of the thyroid is the follicle. It has a diameter of 0.02-0.3mm. The follicle consists of cuboidal cells, which surround a central lumen filled with gelatinous material. In the follicle the thyroid hormones, thyroxine (T_4) and tri-iodothryonine (T_3) are synthesised, stored and secreted. T_4 is synthesised exclusively in the thyroid, while 80% of T_3 is derived from metabolic de-ionisation of T_4 peripherally. T_3 is more biologically active than T_4, and has a short half-life (1day) compared with that of T_4 (6.2 days). In plasma, 99% of thyroid hormone is protein bound.

1.4 Thyroid Hormone Synthesis

The thyroid accumulates iodine by an active transport process that is dependent on ATP. Membrane Na+ K+ ATPase is probably also involved in the transport process. Under iodine deficient circumstances, a thyroid versus plasma gradient as high as 500:1 can be achieved. The transport process follows enzyme kinetics and is inhibited by $TcO_4^-<ClO_4^-<SCN^-<I^-<NO_2$. In clinical medicine use is made of these characteristics in that TcO_4^- (pertechnetate) is employed to visualise the thyroid, and ClO_4^- (perchlorate) to block the thyroid gland, when radioactive pharmaceuticals containing iodine are used, for protection of the thyroid.

The principal stimulator of the iodine pump is serum TSH acting via a specific membrane receptor located in the thyrocyte plasma membrane. Once taken up by the thyroid cell, iodine diffuses freely through the thyroid cell cytoplasm to the apical membrane. Iodination of thyroglobulin takes place near or at this membrane. Iodine is oxidised at this site, involving peroxidase and hydrogen peroxide before it is linked to tyrosine residues present in thryoglobulin.

Thyroglobulin is a specific thyroid protein comprising two different sub-units each with a molecular weight of approximately 330 Kda. Thyroglobulin is especially suited to thyroid hormone biosynthesis due to an abundance of tyrosine molecules (122/molecule). When these tyrosine residues are linked with iodine they give rise to the formation of mono-iodotyrosine (MIT) or di-iodotyrosine (DIT). Subsequent combination of these iodinated tyrosine residues of thyroglobulin lead to the formation of iodothyronines. If DIT combines with DIT 3,3'5,5'-tetraiodothryonine, thyroxine (T4) is produced. Alternatively, if MIT combines with DIT either 3,3',5-triiodothyronine (T3) or 3,3',5'-triiodothryonine reverse T3 (rT3), is formed.

This coupling reaction is catalysed by peroxidase in the presence of H_2O_2. Iodothryonines remain part of the thyroglobulin molecule and are stored in the follicular lumen. Through a process of endocytosis by follicular cells colloid is

taken up in the thyrocyte in the form of discrete intracellular endosomes. These endosomes fuse with hydrolase containing lysosomes. The hydrolases act upon thyroglobulin causing the liberation of T4, T3 and rT3 together with MIT and DIT. Almost all the MIT and DIT is deiodinated and the released iodine re-utilised in the follicle.

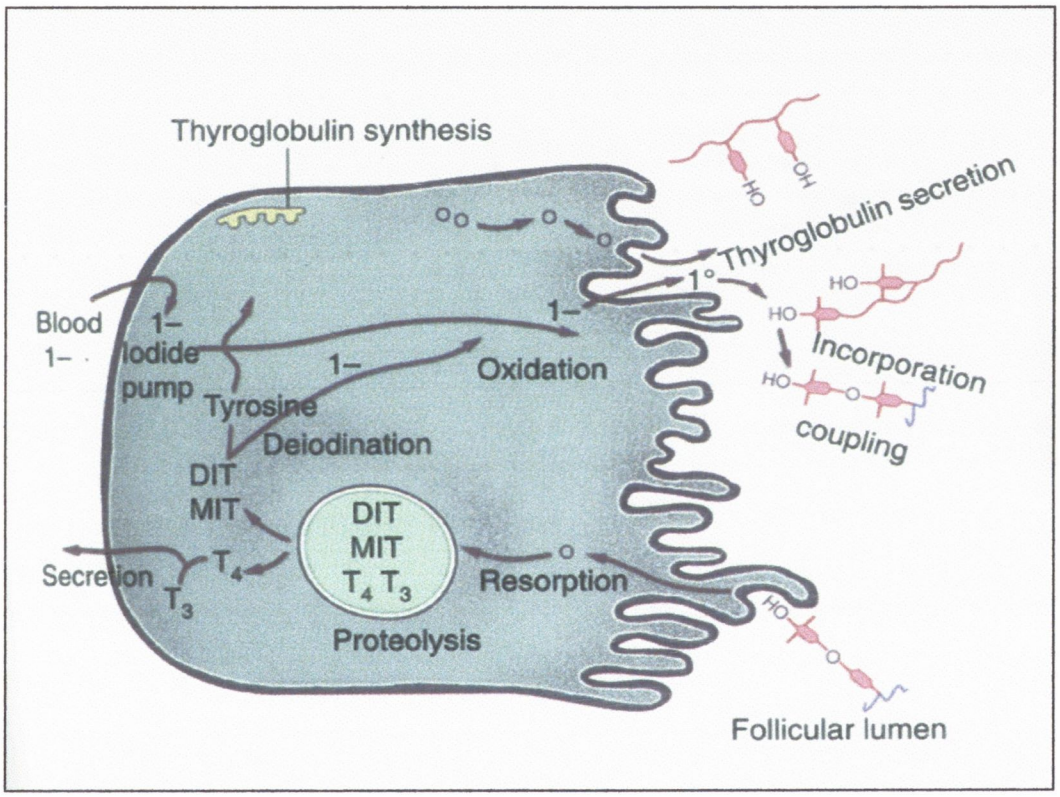

Fig.1.1 Follicle Cell demonstrating synthetic function.

1.5 Regulation of Thyroid Function

The main physiological stimulator of the thyroid gland is thyroid stimulating hormone, thyrotropin (TSH). TSH is a glycoprotein with a molecular weight in the region of 28,000 kDa, which is released from the pituitary gland. It is composed of two non-covalently bound sub-units. The α-sub-unit is common to a number of other peptide hormones such as luteinising hormone (LH), follicle stimulating hormone (FSH) and human chorionic gonadotrophin (hCG). The β-subunit is specific for TSH.

TSH stimulates cyclic AMP by its interaction with a specific plasma membrane receptor. This stimulatory pathway activates all steps in the chain of thyroid hormone synthesis, including the endocytic process for transport of thyroglobulin into the thyroid cells. The production and secretion of TSH is controlled by a variety of stimulators and suppressors. Suppressors include the thyroid hormones in particular T_3 and agents such as dopamine and somatostatin. TSH synthesis and release is promoted by thyrotropin releasing hormone (TRH). TRH is derived from the hypothalamus. Its release is inhibited by thyroid hormone.

Thyroid hormones exhibit low solubility in aqueous solutions and only a small fraction exist in free form in serum (<1%). Thyroid hormones are bound to three main thyroid hormone binding proteins: thyroxine binding globulin (TBG), thyroxine binding prealbumin (TBPA) and albumin.

1.6 Hormone Receptors and Intracellular Signalling

The communication of hormone signals to the interior of the cell can be achieved by either the generation of second messengers at the cell surface, or by hormones crossing the cell membrane, binding to intracellular receptors and subsequently regulating the expression of specific genes. The response of a target cell to a hormone depends upon recognition of the ligand by receptor molecules on or within the cell. The binding reaction between hormone and receptor must therefore be highly specific and selective, with an affinity that corresponds to the physiological concentration range of the hormone.

A number of hormones, most notably the steroid and thyroid hormones, are lipophilic and are therefore able to diffuse through the phospholipid bilayer of the plasma membrane. Whether the receptor is on the cell surface or within the cytosol, the hormone interacts specifically with the receptor, altering its conformation and signalling its presence to the cell.

1.7 The Thyrotropin Receptor (TSHr)

The TSHr is a member of the seven transmembrane segment G protein associated receptors. In addition it has an extracellular domain of some 418 amino acids. There are approximately 1000 receptor-binding sites on the surface of a normal human thyroid cell. Like luteinising hormone (LH)/human chorionic gonadotrophin (hCG) receptor, with which it shares some sequence homology, the receptor is linked to a G-protein and its effects are mediated by cyclic adenosine monophosphate.

The G-protein-associated receptor superfamily are a group of closely related heterodimeric proteins composed of three subunits designated α (39-56kDa), β

(37kDa) and γ (8kDA), which are distinct gene products. TSHr is related to the other G-protein coupled receptors in that it contains seven transmembrane regions. However, in contrast to G-protein coupled proteins that bind small ligands, receptors of this group (i.e. LH/CGr, FSHr and TSHr) have a large glycosylated extracellular moiety that is involved in ligand binding. This characteristic makes pituitary glycoprotein hormone receptors members of a specific sub-family of G-protein coupled receptors.

Moreover, the TSHr is of interest because it can be the target of autoimmune reactions. Autoantibodies directed against the TSHr are directly responsible for the pathogenesis of Graves' disease and idiopathic myxoedema.

1.8 Pathogenesis – oncogenes

Sample availability and difficulty in experimental manipulation of human cells limit knowledge of tumour initiation in human epithelia. The thyroid is a useful model in that, in addition to multiple stages it presents two distinct pathways of tumourigenesis: follicular tumours (FTC) in which *ras* oncogene mutations occur at high frequency, and papillary tumours (PTC) associated with *ret* or *trk* activation. It has been shown that mutant p53 occurs only in the later stages of thyroid carcinoma, hence focus has centred more recently on *ras* and *ret* mutations with the objective of devising earlier markers of tumour initiation.

FTC represent an adenoma – carcinoma sequence analogous to colon, and when malignant metastasise widely via the bloodstream. PTC, in contrast appears to arise directly as carcinoma, although the majority remain as small 'occult' lesions. Those that progress do so via lymphatics and generally carry a better prognosis than their follicular counterparts.

Prognosis in thyroid carcinoma is usually assessed on the basis of criteria which include the patient's age and the histological type, grade and stage of tumour. It is well recognised that while occasional tumours with adverse histological features, such as necrosis or a high mitotic rate, are cured, other seemingly well-differentiated ones may progress to a fatal outcome. Surgical removal is the mainstay of treatment in thyroid cancer and presumably those high-grade tumours which are cured are those of limited spread in which extirpation has been successful. In addition to surgery, in tumours of lower grade, TSH suppression by thyroxine or TSH stimulation in conjunction with I^{131} administration are important medical strategies used to retard the growth of, or to ablate, residual or metastatic disease. It is reasonable to assume that only TSHr rich neoplasms will be susceptible to TSH manipulation and the identification of these variants has significant clinical implications.

1.9 RET proto-oncogene

The molecular mechanisms of malignant transformation in the thyroid gland are not clearly delineated. Papillary thyroid carcinomas represent a unique model among human solid tumours to study the mechanisms of oncogene activation following chromosomal re-arrangements. c-*ret* encodes a receptor type tyrosine kinase with a cadherin-related sequence located in the extracellular domain of the protein (Kuma et al 1993). To date, the ligand of the ret tyrosine kinase has not been determined.

The *ret* proto-oncogene is expressed in a developmental stage specific manner (Tsuzuki et al 1995). Increased expression of RET in combination with germ line mutations has been found in human cell lines and tumours from cells of neural crest origin such as neuroblastomas, phaeochromocytomas and medullary thyroid carcinomas. c-*ret* mutations have been detected in familial medullary thyroid

carcinomas, multiple endocrine neoplasia type 2A and 2B and in Hirschsprung disease.

1.10 RET and PTC

The *ret* transforming gene isolated by Takahashi et al. had been activated *in vitro* during transfection assay where a re-arrangement that juxtaposed two unlinked human DNA segments, the ret proto-oncogene and the putative zinc finger-containing *RFP* gene (*r*et *f*inger *p*rotein).

The RET oncogenes in human papillary thyroid carcinomas result from chromosomal re-arrangements that occur in intron sequences of cRET and other genes. In *vivo*, three different forms of RET activation (*ret*/PTC-1, *ret*/PTC-2 and *ret*/PTC-3) have been identified to date, and their expression has been reported in thyroid carcinomas.

The resultant fusion proteins display a constitutive tyrosine kinase activity, while ligand binding and transmembrane portions of cRET are lost (figure 1.2). The majority of re-arrangements are in the form of *ret*/PTC-1, consisting of the tyrosine kinase domain of cRET and a partial sequence of a gene termed H4 (D10S170). This is a somatic, tumour specific event in contrast to the recombination between *RET* and *RFP*. Alternative splicing of proto-RET gives rise to differing C-termini and corresponding PTC cDNAs have been isolated.

In *ret*/PTC-2 the tyrosine kinase domain of cRET is juxtaposed to the 5' terminal sequence of the regulatory sub-unit *RIα* of cAMP-dependent protein kinase. *ret*/PTC-3 is composed of the tyrosine kinase domain of cRET and the amino terminal of the ELE-1 gene product.

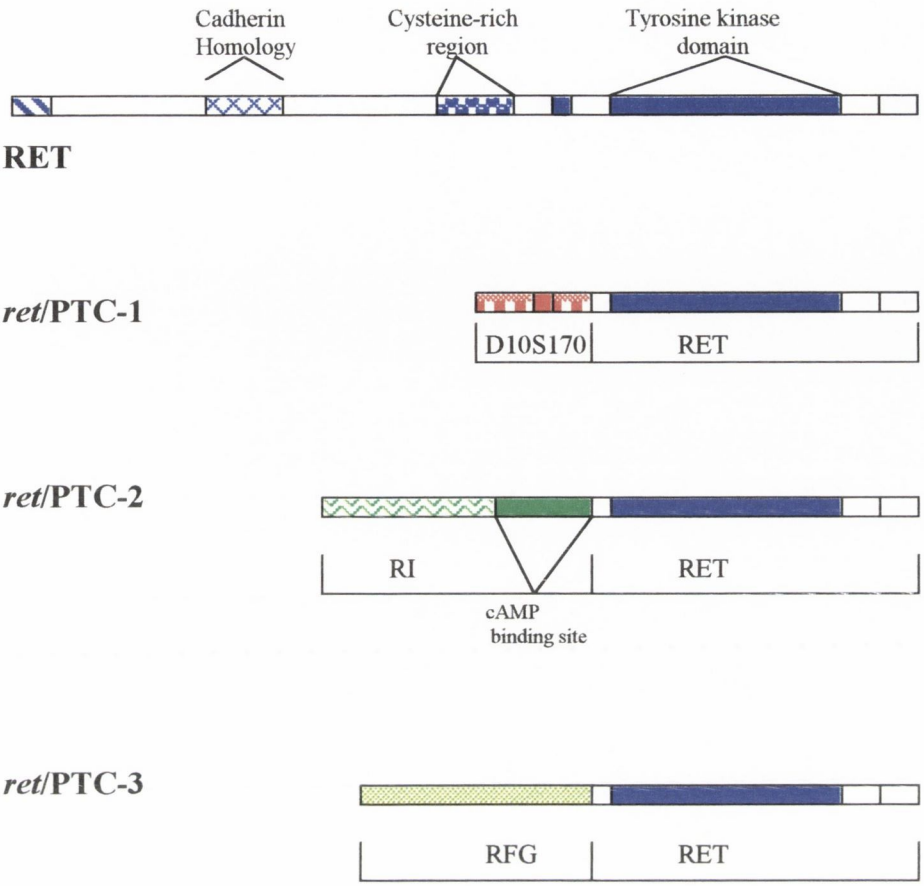

Fig 1.2 **Different Forms of ret activation**

1.11 RAS

Mutations of ras genes have been detected in greater than 30% of human tumours. Three families of RAS proteins have been identified, Ha-RAS, K-RAS and N-RAS, each of which is located in a separate chromosomal region. All three are subject to mutations, although the predominance of some mutations tends to be tumour specific.

Activating point mutations of the *ras* genes have been found in all stages of follicular tumours. The majority of mutations have been detected at residue 61 of H-ras and N-ras genes, although variation in the prevalence of ras point mutation has occurred among different series. These differences have been attributed to environmental factors such as dietary iodine supply. It has been suggested that *ras* mutations represent an early event in thyroid tumourigenesis, and adjacent benign tissue has frequently been shown to exhibit activating *ras* mutations. This emphasises the fact that additional cellular changes are required subsequent to *ras* activation to achieve the malignant phenotype.

1.12 Thyroid Pathology

1.12.1 Thyroiditis

Thyroiditis may present in an acute or chronic form. Acute suppurative thyroiditis may result from local or haematogenous spread of pyogenic bacteria to the thyroid, while chronic inflammation may typically be due to such agents as tuberculosis, syphilis, actinomycosis or fungal infection. The incidence of both these conditions is rare. Conditions including sarcoidosis and rheumatoid arthritis may give rise to non-infective thyroid granulomata, as may trauma (palpation thyroiditis). However, the most common chronic inflammatory condition of the

thyroid is autoimmune thyroiditis. It is characterised by infiltration of the gland by lymphoid cells and epithelial abnormalities.

Three main forms of autoimmune thyroiditis are recognised; Hashimoto thyroiditis, atrophic thyroiditis and focal thyroiditis. Two other forms of thyroiditis are recognised clinically and pathologically and are referred to eponymously as de Quervain's disease and Riedel's disease. The vast majority of patients with autoimmune thyroiditis have circulating antibodies directed against one or more organ specific auto-antigens. These include thyroglobulin and a microsomal antigen closely associated with thyroid peroxidase. These antibodies can be demonstrated by conventional serological techniques, are polyclonal, mainly IgG, and react *in vitro* with the patient's own thyroid antigens. High titre autoantibodies are found most frequently in Hashimoto thyroiditis and less often in focal lymphocytic thyroiditis.

1.12.2 Hashimoto Thyroiditis

This is by far the most common form of thyroiditis, with a manifestation among females some 20 times that of males. Clinically the thyroid is enlarged, firm and painless. The majority of patients are hypothyroid on presentation with elevated serum TSH levels. The cardinal histological features are a massive diffuse infiltration of the thyroid by lymphoid cells and thickening of the fibrous interlobular septa. The lymphoid infiltrate is composed of mature lymphocytes and plasma cells, and lymphoid follicles with germinal centres are generally present. Mitotic figures are seldom encountered, except in the follicle centres. The thyroid follicles are small with scanty colloid. Clusters of follicular thyroid cells frequently have no visible lumen, and the individual cells tend to be large and cuboidal. The cytoplasm has a characteristic granular appearance due to the presence of large numbers of mitochondria. The nuclei may be large, hyperchromatic and irregular in shape. Despite the increased content of

mitochondria within the cells they have impaired iodination ability and diminished production of thyroid hormone.

1.12.3 Atrophic Thyroiditis

This condition is responsible for non-goitrous hypothyroidism or primary myxoedema, which is a common disease in elderly patients. Either sex may be affected, although there is a slight preponderance among females. The thyroid gland is reduced to a fibrous remnant, with no histologically normal thyroid tissue seen.

1.12.4 Focal Autoimmune Thyroiditis

The main defining feature of this condition is the patchy lymphoid infiltrate, which distinguishes it from Hashimoto thyroiditis or atrophic thyroiditis. Patients may have subclinical hypothyroidism with elevated serum TSH levels, and in some cases it represents an early stage of atrophic thyroiditis.

1.12.5 Thyroid Adenoma

Thyroid adenomas are common thyroid lesions, which are usually solitary and have a complete fibrous capsule. Microscopically the epithelial cells are usually uniform in appearance with pale, regularly sized nuclei, inconspicuous nucleoli and scant mitoses. The cells may be arranged as solid trabeculae (embryonal adenoma), minute follicles (foetal adenoma) or masses of small follicles containing colloid (follicular adenoma). These variations are of no clinical significance. Most follicular adenomas (FA) take up less radioiodine than the surrounding normal thyroid and, on scanning, appear as 'cold nodules'. The malignant counterpart of FA is follicular thyroid carcinoma (FTC).

1.13 Malignant Tumours of the Thyroid Follicular Cell

1.13.1 Papillary Carcinoma

Papillary carcinoma of the thyroid (PTC) is the most common thyroid malignancy, accounting for approximately 80% of cases. PTC generally presents in the fourth or fifth decade with a female: male ratio of 3:1.

In general PTC is a well-differentiated, indolent disease with good prognosis. PTC often has ill-defined margins and may be multi-focal. Microscopically it is best defined by the presence of papillae and nuclear changes. The papillae are composed of a central fibro-vascular core suffused by an epithelial lining. The papillae may adopt a variety of patterns and the follicular cells may be columnar or cuboidal.

The nuclei of PTC have several distinctive features. One such characteristic is the finding of cytoplasmic invaginations into the nuclear membrane, which result in nuclear pseudo-inclusions and grooves. Another - ground glass or orphan Annie nuclei -occurs as a result of the nucleolus being pushed to the side, giving the impression of 'empty' nuclei. Mitoses in PTC are uncommon, and if present tend to be associated with de-differentiation.

Psammoma bodies are seen in approximately 50% of PTC. They are calcific concretions with concentric laminations, and while associated with PTC they may also occur in medullary thyroid carcinoma.

Significant poor indicators of prognosis in PTC include increased age and male sex. Tumours >5cm in size and those with extraglandular spread have increased rates of recurrence. These tumours tend to spread through the lymphatics within the thyroid to the regional lymph nodes and more rarely to the lungs.

1.13.2 Radiation Induced Thyroid Carcinoma

Thyroid cancer accounts for approximately 1.5% of cancer deaths, except in the case of exposure to ionising radiation, when the incidence is significantly increased.

On 26 April 1986 the most serious accident in the history of the nuclear industry occurred at the Chernobyl nuclear power plant in the former Soviet Union, near the present borders of Ukraine, Belarus and Russia. As a result of the accident, the reactor was damaged and, over the ensuing 10 days, large quantities of radionuclides were ejected into the environment.
More than ten years after the Chernobyl accident, the highly significant rise in thyroid cancer in those exposed as children in the three most affected countries is

the only evidence to date of a public health impact of radiation exposure as a result of the accident.

As a result of the accident Belarus received 70% of the radioactive fallout. The incidence of thyroid cancer has increased by 2,400 per cent. It is now estimated that one out of every four babies will contract cancer of the thyroid gland. Analyses by cohort at age of exposure confirmed the hypothesis that very young children were at the greatest risk. It is believed that there may be a continuing increase in the incidence of thyroid cancer particularly in those exposed as young children.

Radioiodine was one of the major radioactive components released by the reactor. The fact that the thyroid concentrates iodine supports the concept that one or more radioactive isotopes of iodine are the causative agents. At presentation the majority of tumours have been in an advanced stage, with extension to tissues outside the thyroid gland and lymph node metastases. The pathology of virtually all the cases shows PTC, many with an unusual solid/follicular pattern of growth.

1.13.3 Follicular Carcinoma

Follicular carcinoma of the thyroid (FTC) is characterised by follicular differentiation but without the nuclear changes characteristic of PTC. FTC tend to be encapsulated, and invasion of the capsule and vessels is the key feature distinguishing FTC from follicular adenomas (FA). FTC accounts for approximately 5% of thyroid malignancies, although the incidence of FTC is dramatically increased in areas of iodine deficiency.

Two types of FTC are recognised according to their pattern of invasion: minimally invasive and widely invasive carcinomas. The growth pattern may also vary, ranging from a well-differentiated pattern with macrofollicular structures to a

poorly differentiated pattern with areas of solid growth and a high degree of atypia. Hürthle-cell (oxyphilic follicular or oncocytic) carcinoma is a cytologic variant of FTC. In these neoplasms, the cytoplasm of the oncocyte is crowded with mitochondria, resulting in an enlarged, finely granular, eosinophilic appearance.

Multicentricity and lymphnode involvement are less common than with PTC, and metastases to the lungs and bones stem from haematologic spread.

1.13.4 Anaplastic Carcinoma

Anaplastic carcinoma of the thyroid (ATC) accounts for $\leq$ 5% of thyroid malignancies, is highly aggressive, and is nearly always fatal. Extrathyroidal invasion and metastases are common. Microscopically, a variety of patterns may be seen including epithelial, spindle and giant cell variants. The spindle cell regions resemble sarcomas, while giant cell areas contain large pleomorphic cells with bizarre, often multiple, nuclei. Mitoses are generally numerous with abundant abnormal configurations. Necrosis and vascular invasion are common. Examination of the excised tumour often reveals areas of previously well-differentiated thyroid carcinoma.

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CHAPTER 2

METHODOLOGY

2.1 Introduction

This chapter is a comprehensive account of all the methodologies employed in this thesis, along with background information for several of the newer techniques. Some of the techniques are used in a number of chapters e.g. TaqMan RT-PCR. Where this occurs, the specific primers and probes for each assay are indicated in the body of the relevant text, but the full description of the technique is restricted to this chapter only.

The descriptions of each technique are grouped in sections (where the methodologies are linked) and are listed roughly in the order in which they appear in the text of the thesis.

2.2 Section 1

2.2.1 Immunohistochemistry

ABC (elite) method.

1. Cut 4μm sections onto slides coated with 3-aminopropyltriethoxysilane (APES).
2. Dry section overnight @ 37^0C.
3. Dewax sections in two washes of xylene 5 mins each
4. Rehydrate sections in decreasing concentrations of ethanol – 100%→ 80%→60%→40%→H_2O.
5. [Unmask antigens (Cattoretti 1992) for Ki-67 and p53 antibodies, using 0.01M Citrate buffer (pH6) and microwave treatment.]
6. Block endogenous peroxidase with 0.1% H_2O_2 in Methanol x 30 mins.
7. Block non-specific binding with 0.1% BSA in TRIS buffer.
8. Apply primary antibody at pre-optimised concentration x 60 mins.
9. Wash slides in TRIS saline x 10 mins.
10. Apply biotinylated secondary species specific antibody x 30 mins.
11. Wash slides in TRIS saline x 10 mins.
12. Apply ABC streptavidin reagent x 45 mins.

13. Wash slides in TRIS saline x 10 mins.
14. Apply chromagen reagent (di-aminobenzidine (DAB)) x 10 mins.
15. Wash slides in TRIS saline x 10 mins.
16. Wash slides in running water.
17. Counterstain with Mayer's Haematoxylin x 30 seconds.
18. Blue sections in running water x 15 mins.
19. Dehydrate sections through graded ethanol 40%→60%→80→100%.
20. Clear sections in Xylene and mount in DPX.

Note:

- Primary antibodies are made in TRIS buffer.
- Dilution for biotinylated 2° antibody is 1:300 in TRIS buffer.
- The ABC reagent must be made up 30mins before use.

2.2.2 Antibodies used and Optimised Dilutions

Antibody	Type/species	Dilution
Ki-67	Polyclonal/rabbit	1:70
p53 (DO7)	Monoclonal/mouse	1:100
EGFR	Monoclonal/mouse	1:10
calcitonin	Polyclona/rabbit	1:150
thyroglobulin	Polyclonal/rabbit	1:1000

2.2.3 Antigen Unmasking

Following step 4 in ABC method (above), proceed as follows:

1. Transfer slides to 900ml of 10mM citrate buffer* in a plastic beaker. Cover the container with clingfilm. Puncture clingfilm several times and place in microwave oven at full power x 20mins.

2. Remove beaker form the oven and allow to stand at room temperature x 15 mins.
3. Rinse in tris buffer and proceed with ABC staining protocol.

*Citrate Buffer

2.1g citric acid monohydrate in 800ml distilled H_2O. Adjust the pH to 6.0 with approximately 13ml of 2M NaOH. Make up to 1L with distilled H_2O.

2.2.4 Coating slides with 3-amino-propyl-triethoxysilane (APES)

- Immerse slides in 2% Decon 90 x 30 mins.
- Wash slides thoroughly in distilled water to remove detergent.
- Drain off excess water and place slides in 100% alcohol x 2mins.
- Drain off excess acohol and place slides in 2% APES in alcohol x 5ins
- Drain off excess and rinse slides in 100% alcohol.
- Wash slides in running water x 15 mins.
- Drain off excess water and dry slides in hot air oven.
- Store slides in a dust free box @ RT until needed.

2.3 Section 2

2.3.1 Tissue Culture of TPC-1 Cells

The TPC-1 cell line is known to express the *ret*/PTC-1 chimeric transcript, and thus it was selected as a source of positive control RNA for the assessment of RET activation in this study.

Expression of differentiated epithelial cell functions depends on formation of a confluent culture and cell polarisation. Such differentiation is supported by culturing onto a supporting medium, plating at high cell density, exposure of the apical cell surfaces to humidified air, and the use of differentiation inducing media. In these conditions, the cells achieve optical confluence 2-3days after plating.

TPC-1 cells were grown to confluence in the following plating medium:

Dulbecco's Modification of Eagle's Medium-F12 (DMEM F12)	9.ml
Foetal Calf Serum	1ml
TSH	10mU/ml
Insulin	0.01mg/ml
Penicillin	100U/ml
Streptomycin	100µg/ml

When the cells attained confluence, they were trypsinised and sub-cultured or used for RNA extraction or *in-situ* RT-PCR.

2.3.2 Long term culture and passage

Materials

Trypsin/EDTA: 0.1% trypsin type III (Sigma T0646) + 1mM EDTA (Sigma E6635) in phosphate-buffered saline (PBS)

Soybean trypsin inhibitor (STI, Sigma T9128)1mg/ml in F-12

Protocol for trypsinisation

- Aspirate medium and rinse cells with sterile PBS.
- Add trypsin/EDTA, about 50µl/cm^2, just enough to cover all the cells.
- Incubate @ 37°C.
- Examine by phase microscopy every 2-3 mins to assess cell detachment. Detach rounded cells by tapping the flask.
- When >90% of cells are detached, add an equal volume of STI, to inhibit the trypsin.
- Collect and pellet cells (500*g*, 5 mins).
- Resuspend the cells at ~ 1x10^6/ml of plating medium. Count an aliquot in a haemocytometer.
- Plate on fresh tissue culture plates at 1:3 or 1:4 dilution.

2.3.3 Cryopreservation

Cultured cells can be frozen and stored for months-years by the addition of cryopreservative agents that inhibit the formation of ice crystals.

Freezing Medium:

DMEM	7ml
Foetal Calf Serum	2ml
DimethylSulphoxide (DMSO)	1ml

Protocol for freezing

- Trypsinise cells and re-suspend in culture medium at a density of 4-6x10^6 cells/ml. Place on crushed ice.
- Add an equal volume of freezing solution drop by drop over 5 mins. Mix by shaking intermittently.
- Aliquot 2-3x10^6 cells (1ml) to labelled cryovials.

- Put vials into an insulated Styrofoam box and place in –70°C freezer overnight to permit slow cooling.
- Transfer vial to liquid nitrogen storage tank for long term storage.

Protocol for thawing

- Remove cryovial from liquid nitrogen and thaw in a beaker of 37°C water, keeping the cap dry.
- As soon as cells have thawed, add 1ml of prewarmed plating medium to the cryovial and gently transfer the cells to an empty 15ml tube. Add 10ml of prewarmed plating medium dropwise over1-2 mins to dilute the DMSO.
- Pellet @ 500*g* for 5 mins.
- Resuspend cells in plating medium and plate at relatively high (~$8x10^4$ cells/cm^2) seeding density.

2.4 Section 3

2.4.1 Quantitative PCR (Q-PCR)

The direct application of tests based on nucleic acid analysis to the diagnosis of genetic alterations without amplification has the disadvantage of low sensitivity. Nucleic acid amplification techniques such as polymerase chain reaction (PCR) increase sensitivity dramatically while maintaining specificity. However, the characteristics of conventional PCR amplification do not allow the quantitation of nucleic targets present in the initial sample.

In recent years, several approaches have been proposed that attempt to perform PCR based quantitative assays. The strategy for Q-PCR must be accurately selected based on the quantitative requirements, including the methods of quantitation of the amplicons and the type of standard used in the assay. Different

quantitative approaches can be followed based on the use of internal or external standards.

Relative quantitation is usually all that is required and is used to compare differences in targets among different samples. This can be done by assaying a reference gene separately or together with the unknown target and assessing the level of expression as a simple ratio. Another strategy is to use a calibration sample, a technique which allows the evaluation of a biological effect in comparison to a basal level, and is commonly applied to *in vitro* studies.

Absolute quantitation determines the exact number of copies of a specified target in a sample. There are a number of technical considerations to this technique, such as matching the reference substance and target as closely as possible and accurate quantitation of the reference material.

2.4.2 Problems with quantitative PCR

The main factor in attaining successful quantitative PCR is optimisation of the amplification process. It is crucial that non-specific hybridisation effects, which occur in the early cycles of PCR and may lead to erroneous results are reduced to a minimum. To this end 'hot-start' PCR is now commonly used.

The linear range of amplification is another vital consideration in the development of quantitative PCR assays. In theory, if the amplification proceeds with 100% efficiency, the amount of amplicons will double with each cycle. However, in most PCR assays the overall efficiency may be as low as 70-80% during the exponential phase of the reaction. The plateau phenomenon occurs due to limiting factors including substrate saturation of the enzyme, incomplete product strand separation and exhaustion of the reaction components. Consequently, the exponential increase in amplicons only occurs for a limited number of cycles before a plateau is reached. In the plateau phase of a PCR reaction, the amount of product formed is no longer proportional to the amount of starting material. It is essential that measurements be made only during the exponential phase if any degree of accuracy in quantitation is to be achieved.

2.4.3 Relative Quantitation

Relative quantitation is a common procedure used to assess differences in target sequences among different samples. It is particularly relevant in the assessment of functional variations in mRNA molecules. In general, the signal for the target sequence to be estimated is referred to a ubiquitously expressed house-keeping or reference gene.

2.4.4 Reference Genes

A reference gene can be assayed separately or together with the unknown target and their final ratio calculated. The reference gene can be an endogenous or exogenous mRNA target. In both cases this strategy has the advantage of compensating for the high variability in the efficiency of the RT reaction.
The use of an endogenous reference gene can also prevent errors which arise due to inaccurate estimation of total RNA concentration and quality in the original sample. The expression of a housekeeping gene (β-actin, GAPDH, β_2-microglobin, ribosomal RNA etc.) is commonly used as an endogenous reference mRNA target. However, caution must be exercised in the selection of a housekeeping control gene, as expression can be variable among different tissue types or even cell-cycle steps depending on the reference gene selected. In addition, expression of the housekeeping gene may be very high compared with that of the unknown target, and in such a situation, the efficiency of amplification between the two targets can differ considerably.

Alternatively, an exogenous target mRNA may be co-amplified with the target, with the advantage that the quantity of the reference gene can be adjusted to fixed concentrations within the range of the unknown target. A potential drawback to this rationale is that alterations in quality of mRNA in the initial sample which are inevitable in archival material are not taken into consideration and may lead to erroneous results.

2.4.5 TaqMan Chemistry

TaqMan is a technique which utilises a characteristic of AmpliTaq and AmpliTaq Gold DNA polymerases, namely their Y-shaped structure dependent, polymerase associated, 5'-3' endonucleolytic activity. It allows for the direct detection of PCR product by the specific release of a fluorescent reporter molecule during the PCR reaction. TaqMan PCR utilises a primer pair (as in conventional solution phase PCR) and an internal oligonucleotide probe, called a TaqMan probe.

The TaqMan probe comprises an oligonucleotide 20-30 bases in length with a 5' reporter dye, a 3' quencher dye, and a 3' blocking phosphate. The most commonly used fluorescent reporter dye is FAM (6-carboxyfluorescein) which is covalently linked to the 5' end of the oligonucleotide probe. Alternative reporter dyes are TET (tetrachloro-6-carboxy-fluorescein) and HEX (hexachloro-6-carboxyfluorescein). Each of these reporters is quenched by TAMRA (6-carboxy-tetramethyl-rhodamine) which is attached by a LAN (linker-arm-modified nucleotide) to the 3'end of the probe. The probe is chemically phosphorylated at its 3' end, which prevents probe extension during PCR cycling.

When the probe is intact (i.e. linearised), the proximity of the reporter dye to the quencher dye results in direct suppression of fluorescence from the reporter dye by Förster-type energy transfer. During PCR, if the target of interest is present the probe will specifically anneal between the forward and reverse primers. The probe will be cleaved due to the nucleolytic activity of AmpliTaq DNA polymerase if the probe is hybridised to its target. The subsequent release of the fluorescent reporter will only occur if target specific amplification occurs, obviating the need to confirm the amplicon following amplification.
Importantly, Taq DNA polymerase does not digest free probe. After cleavage, the shortened probe dissociates from the target and strand polymerisation continues. This process occurs every cycle and does not interfere with the exponential accumulation of product.

The specificity of the 5' nuclease assay results from the requirement of the Taq DNA polymerase enzyme for sequence complementarity between the probe and the template, in order that specific cleavage of the probe occurs.

2.4.6 End point Detection

For end-point detection, a luminescence spectrometer is used (ABI Prism 7200). The increase in fluorescence is compared to the fluorescence of a 'no template control (NTC)'. To normalise for pipetting errors and volume changes that inevitably occur during PCR, the reporter fluorescence is divided by the quencher fluorescence to yield a ratio termed the RQ for each reaction.

The difference between the sample RQ (RQ+) and the NTC RQ (RQ-) is called the ΔRQ. This difference represents the amplification of a specific product, which has occurred during PCR.

To assure a statistically high confidence level in the results generated by TaqMan PCR, the protocol should include a minimum of three NTCs. Based on T distribution values (Beyer 1984) any ΔRQ value above the threshold ΔRQ has a 99.7% confidence level of being a positive result.

2.4.7 Real Time Detection

Using the real time detector (ABI Prism 7700), a value called the ΔRn is obtained. The 7700 detector system collects fluorescent emissions between 500 and 600 nm from each of 96 sample wells, once every 7 seconds. The emissions are collected through fibre optic cables (which are positioned directly above each of the 96 sample well positions), focussed by a dichroic mirror onto a spectrograph. The light is separated according to wavelength by a CCD camera and the data analysed by the software's algorithms. The 7700 system utilises specially designed reaction tubes that remain closed throughout the PCR amplification and detection process, thus reducing the chances of cross-contamination.

The main difference between the 7200 and 7700 detectors is that the 7700 instrument monitors PCR every cycle. Quantitative data is generated based on the PCR during early cycles, when the PCR fidelity is at its highest. The 7700 has a linear dynamic range of at least five orders of magnitude, obviating the need for serial dilutions.

During the PCR reaction, the emission intensities of both the reporter and quencher dyes are evaluated. The intensity of the quencher remains relatively constant throughout the PCR process, and as such it is used to normalise variations in reporter emission intensities (figure 2.1). The software calculates a value termed ΔRn according to the following equation: $\Delta Rn = (Rn^{+}) - (Rn^{-})$, where Rn^{+} is the emission intensity of the reporter/quencher at any given time in a reaction tube, and Rn^{-} is the emission intensity of the reporter/quencher prior to amplification in the same reaction tube.

A TaqMan amplification plot typically adopts a sigmoidal shape. In the initial stages, there is insufficient reporter cleaved to elevate the emission intensity above the baseline. After a certain number of cycles (C_T), probe cleavage is such that reporter emission intensity rises above the baseline. The C_T is dependent on the starting template copy number, the efficiency of amplification and the cleavage of the TaqMan probe. An inverse relationship is observed between the amount of template present and the threshold cycle for that reaction. This is logical, because reactions with fewer starting copies of the target molecule require a greater number of amplification cycles to generate a detectable signal. Consequently, C_T measurement can be used as a quantitative indicator of the input target number.

2.4.8 Standards and Quantitative PCR.

In order to assess the linear range of amplification, standards of known concentration and similar composition to the target should be employed. It is

noteworthy that a DNA standard is not the optimal choice when dealing with an RNA assay system. With increased interest in functional genomics, assays, which rely on the measurement of RNA expression of a specified target, are gaining in popularity. In such an assay, DNA would be inappropriate as a standard, as it would not take into account inter-sample variations due to the reverse transcription step. The standard of choice in RNA assays is the use of cRNA (copy RNA), or at least RNA of known concentrations. These standards can be generated by amplifying a sequence somewhat larger than the TaqMan target. A set of outer primers is used to this end. The derived amplicon may be purified and cloned into a suitable vector and subsequently purified.

2.4.9 Amplification Plot

An amplification plot is the plot of the fluorescence signal versus the cycle number. In the initial cycles of PCR, there is little change in the fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline. The parameter C_T (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. As shown by Higuchi *et al*, a plot of the log of the initital target copy number for a set of standards versus C_T is a straight line. Quantitation of the amount of target in unknown samples is accomplished by measuring C_T and using the standard curve to determine starting copy number.

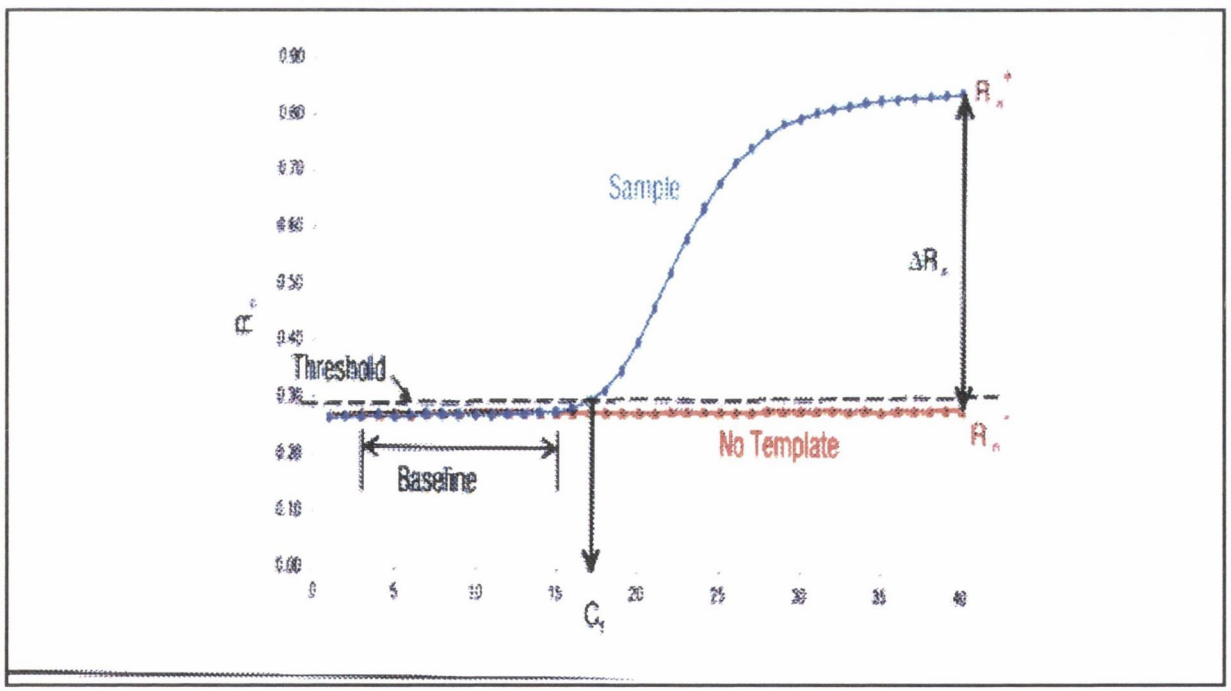

Figure 2.1 Model of a single amplification plot, showing terms commonly used in real-time quantitative PCR.

2.4.10 7700 optics

The 7700 system collects the fluorescent emission between 500-660nm in each of 96 sample wells every 7 seconds. A laser directed to the sample vials through the fibre-optic cable excites the fluorescent dyes (figure 2.2). These fibre-optic cables then carry the fluorescent emissions back to a CCD camera, where they are detected according to their individual wavelengths.

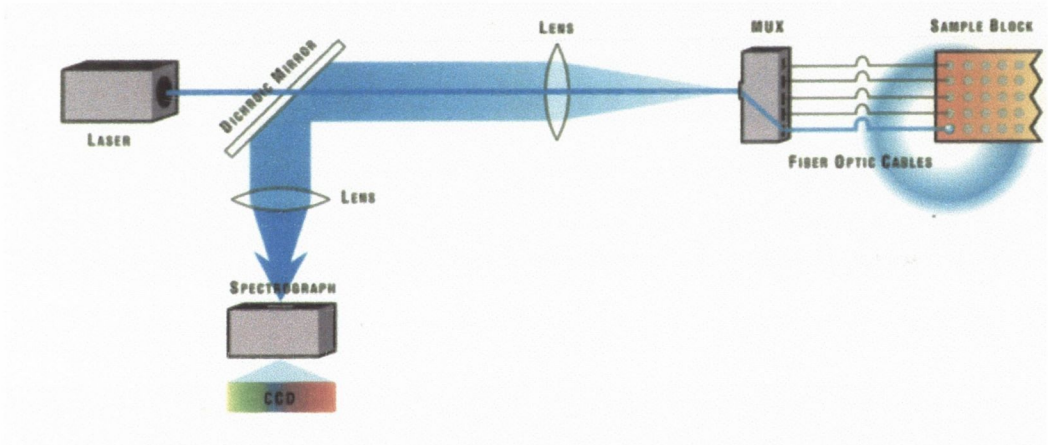

Figure 2.2 7700 optics system.

2.4.11 Heated Lid Assembly

The 7700 system uses specially designed reaction tubes that remain closed throughout the PCR amplification and detection process, reducing the chance of contamination.

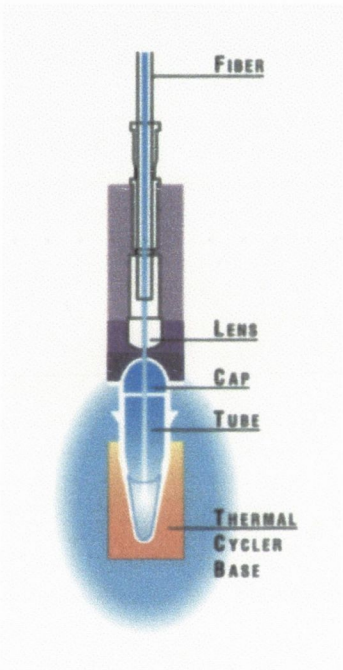

Figure 2.3 Diagrammatic representation of heated lid assembly.

2.5 Section 4

2.5.1 Extraction of RNA from archival Material

The importance of this step cannot be overstressed. If RNA amenable to reproducible amplification cannot be extracted then no amount of sophisticated technology will provide quantitative information about gene expression.
In this study, several extraction protocols were evaluated to determine which was simple enough to allow a realistic rate of sample throughput, while at the same time producing consistently good yields of RNA for amplification.

One of the primary uses of RNA isolation is the analysis of gene expression. In order to elucidate the regulatory properties of a gene, it is necessary to know the structure and quantity of RNA that it produces.
The ability to isolate clean, intact RNA is important in a variety of molecular biological techniques, and is essential to analysing gene expression. Ribonucleases, however, cause difficulty with RNA isolation because they are very stable, active enzymes that require no cofactors to function. Lysis of cells in an environment that causes denaturation of ribonucleases is therefore an essential feature of RNA isolation protocols. RNA is then fractionated from the remaining cellular components.

The extraction protocols evaluated in this study were as follows:

- Guanidium iso-thiocyanate lysis followed by phenol/chloroform extraction.
- RNAzol (Commercial Kit)
- Trizol (Commercial Kit)
- Qiagen (Commercial Kit)
- PureScript (Gentra systems) (Commercial Kit)

Most extraction protocols produce total RNA, composed mainly ribosomal RNA (rRNA) and transfer RNA (tRNA) with a minority of messenger RNA (mRNA).

For the purposes of RT-PCR total RNA is generally acceptable, as the loss of mRNA incurred by purification to remove the rRNA and tRNA may outweigh the advantages of having a pure transcript of mRNA.

The extraction of poly-$(A)^+$ RNA is not an advantage when dealing with archival specimens. There will be degradation of RNA in any archival specimen, and the aspiration that a target transcript may be extracted with its poly-$(A)^+$ tail intact is optimistic in the extreme. In reality, it is highly unlikely that RT-PCR will be successful from poly-$(A)^+$ RNA extracted from an archival specimen due to spatial considerations. Normally, when amplifying from an RNA target, the primers are chosen from two different exons to prevent amplification of any contaminating genomic DNA. In many cases the final exon of RNA transcripts (adjacent to the poly-$(A)^+$ tail) is long (>1000bp), which means for primers to span two exons the amplicon would have to be longer than is reasonable to achieve from a degraded sample.

The major source of failure in any attempt to produce RNA is contamination by ribonuclease. Even the smallest amount of RNase in an RNA preparation will cause severe problems. To avoid contamination, it is essential to observe the following laboratory practices:

- Solutions. Any water or salt solutions used in RNA preparation should be treated with diethylpyrocarbionate (DEPC). DEPC inactivates ribonucleases by covalent modification. It is important to note that any solutions containing TRIS cannot be effectively DEPC treated as TRIS reacts with the DEPC inactivating it.
- Glassware and Plastics. Labware used in the preparation of RNA should be treated to remove residual RNase activity. Glassware can be DEPC treated and then autoclaved, and baked for ~4hours.
- Hands are a major source of RNases, thus gloves should always be worn when dealing with RNA, and they should be changed regularly.

- Pipettes. Aerosol resistant pipette tips should always be used when handling RNA. In addition, pipettes should be UV treated to break down any contaminating nucleic acids that may be harboured on the plastic surface.

2.5.2 Homogenisation of tissue samples.

While evaluating a series of extraction protocols, it became evident that the single most important step was homogenisation of the tissue sample. If the tissue is not homogenised quickly and the lysis solution exposed to all cells in the tissue, then RNA will be degraded before it can be extracted. The forms of homogenisation evaluated were:

- Graded bore needles and syringe
- Mortar and Pestle
- Ultrasound
- Qiagen Shredders
- Tube Pestles

2.5.3 Needle and Syringe

This technique was applied to tissue samples suspended in a 4M Guanidiun isothiocyanate solution. The concept here was to lyse and simultaneously inactivate RNases. Starting with a wide bore needle, the suspension was aspirated into a syringe barrel and expelled several times. The needle was then changed to one with a smaller bore, and the process repeated. The main problem with this technique was the danger of forcing a toxic solution through narrow bore needles. Considerable force was required to push the solution through the needle, and the lysate frequently splashed and was lost.

2.5.4 Mortar and pestle

This method was evaluated with Guanidium isothiocyanate, RNAzol and Trizol protocols. Basically, it involved crushing the tissue in a suspension of lysis solution. The main drawback with this technique was that the friction generated in grinding the tissue had deleterious effects on the quality of RNA extracted. Consequently, successful RT-PCR amplification was not consistent. To overcome

this problem, grinding was attempted under liquid nitrogen. The mortar and pestle were cooled with liquid nitrogen. The tissue which had been de-waxed was placed into the chilled mortar, lysis solution was added and the pestle was used on the frozen suspension. This procedure yielded reasonably consistent quality of RNA from archival material. However, the need to DEPC treat the mortar and pestles and autoclave them prior to use made this technique somewhat cumbersome.

2.5.5 Ultrasound

The technique of sonication was applied to tissues suspended in lysis solution to gently homogenise the tissue samples. This technique was not considered viable as the head attachment of the ultrasound instrument had to be DEPC treated and autoclaved for each sample. Thus, the sample throughput with this technique was extremely limited.

2.5.6 Qiagen Shredders

Qiagen shredders are a spin column based shearing system which are commercially available. They are supplied sterile and RNase free, which is a considerable advantage. However, for the purposes of extraction of RNA from tissue samples they have one major disadvantage in that the tissue tends to get stuck on the top of the column. After centrifugation, the tissue could be seen at the top of the column, and the RNA yield was too low for reproducible amplification.

2.5.7 Tube Pestles

These are a cheap, and readily available. Tube pestles comprise plastic rods which fit exactly into a 1.5ml eppendorf type tube. They can be DEPC treated and autoclaved in bulk, and so sample through put is not an issue with this technique. They worked effectively with both Guanidium isothiocyancate based protocols and the PureScript kit.

The PureScript extraction kit differs from the Guanidium based extraction protocols in that it utilises a high salt precipitation protocol. As Guanidium is a

toxic chemical, and the yield and quality of RNA extracted using tube pestles was comparable with both types of technique, the PureScript system was selected for RNA extraction from formalin fixed paraffin embedded tissue.

2.5.8 Sample Selection

1. With reference to H&E stained sections, select an appropriate area of tumour.
2. The selected area from the slide is matched to the corresponding paraffin embedded (PE) block.
3. The perimeter of the selected area is scored with a sterile scalpel.
4. Cut a single 20 µm section of the selected area into a sterile 1.5 ml microfuge tube.

2.5.9 Sample De-paraffinisation

1. Add 500µl of xylene to the microfuge tube, and incubate x 5 minutes with constant mixing at RT°C.
2. Centrifuge the tube at 13,000-16,000g for 3 minutes to pellet the tissue, and discard the xylene.
3. Repeat steps 1 and 2 twice for a total of three xylene washes.
4. Add 500 µl of 100% ethanol to the tube, and incubate x 5 minutes with constant mixing at RT°C.
5. Centrifuge the tube at 13,000-16,000g to pellet the tissue, and discard the ethanol.
6. Repeat steps 4 and 5 for a total of two ethanol washes.

2.5.10 Cell Lysis

1. Add 300 µl of cell lysis solution. Homogenise the tissue using ≈20 strokes with a microfuge tube pestle.
2. Add 1.5µl RNase-free Proteinase-K (20mg/ml).
3. Mix the sample by inversion 25 times.
4. Incubate the lysate overnight at 55°C with constant mixing, and then cool to room temperature.

2.5.11 Protein-DNA precipitation

1. Add 100µl of Protein-DNA precipitation solution to the cell lysate.
2. Invert the tube gently x 10 and place in an ice bath x 5 minutes.
3. Centrifuge the sample at 13,000-16,000g x 3 minutes to form a tight pellet of the precipitated proteins and DNA. If the pellet is not tight, repeat the centrifugation step.

2.5.12 RNA precipitation

1. Decant the supernatant containing the RNA into a clean 1.5 ml microfuge tube containing 300µl of ice-cold isopropanol, (leaving behind the precipitated protein-DNA pellet).
2. Add 1µl glycogen (20mg/ml).
3. Mix the sample by inversion 50 times.
4. Centrifuge the sample at 13,000-16,000g x 3 minutes; depending on the yield, the RNA may be visible as a small translucent pellet.
5. Pour off the supernatent and drain the tube briefly on clean absorbant paper. Add 300µl 70% ethanol and invert the tube several times to wash the RNA pellet.

6. Centrifuge the tube at 13,000-16,000g x 1 minute.
   Carefully decant the ethanol.
7. Invert the tube and drain on clean absorbant paper and allow to air dry x 10-15 minutes.

2.5.13 RNA Hydration.

1. Place tubes on ice and add 25µl RNA hydration solution (or DEPC water).
2. Hydrate RNA x 30 minutes in an ice bath. Alternatively, the RNA may be stored at -70 to –80°C until use.
3. Before use vortex the sample vigorously and pulse spin. Pipette the sample up and down several times to mix samples further.
4. Store RNA samples at –70 to –80°C

2.5.14 Reverse Transcription Reaction

Reverse transcriptases are derived from retroviruses such as avian myeloblastosis virus (AMV) or Moloney murine leukaemia virus (MMLV), which use them to make DNA copies of their RNA genomes. The AMV enzyme is purified from isolated AMV virus (Verma 1977), whereas the MMLV enzyme is purified from overproducing E coli cells containing the cloned gene (Roth et al 1985). AMV and MMLV are multifunctional enzymes, but are mainly used for RNA directed DNA synthesis. Specifically, deoxyoligonucleotides (either oligo (dT) polymers, random DNA hexamers or a specific primer sequence) are used to initiate extension on an RNA template. The DNA derived from the RNA template is termed complementary DNA (cDNA).

For the applications used in this project random hexamers were chosen as the primer of choice for reverse transcription (RT). Oligo (dT)s were found unsuitable due to RNA degradation in archival material. The limitation with specific primer RT is that the cDNA obtained can only be used for a given PCR

reaction, whereas there is more versatility when the reaction is primed with random hexamers.

2.5.15 Master mix for reverse transcription:

COMPONENT	VOLUME	FINAL CONCENTRATION
25mM $MgCl_2$ Solution	4µl	5mM
10x PCR Buffer 11	2 µl	1x
dATP (2.5mM)	0.5 µl	0.2mM
dTTP (2.5mM)	0.5 µl	0.2mM
dCTG (2.5mM)	0.5 µl	0.2mM
dGTP (2.5mM)	0.5 µl	0.2mM
RNase Inhibitor	1 µl	1U/µl
MuLV Reverse Transcriptase	1 µl	2.5U/ µl
Random Hexamers	1 µl	2.5 µM
RNA	1-9 µl	

Total reverse transcription volume including sample RNA = 20 µl (the balance to be made up with 18Ω dH_2O

Place tubes in thermocycler

1. Incubate tubes at RT x 10 minutes
2. Incubate tubes at 42°C x 30 minutes
3. Denature samples at 99°C x 5 minute
4. Cool samples to 5°C x 5 minutes.
5. The derived cDNA may be used in solution phase or TaqMan PCR.

2.6 Primer Express

- Primer Express (PE-Biosystems) is a software package available for the design of primers and probes.
- Import DNA/cDNA sequence into PrimerExpress.
- Label intron/exon boundaries (if dealing with cDNA).
- Select 'find primers/probes now'
- Choose primer and probe sets so the probe traverses the intronic junction where possible (to eliminate potential signals from any contaminating DNA) according to the following manual criteria.

2.6.1 TaqMan Probe and Primer Design

Primer

- Tm 58°-60°C
- 20-80% GC content
- Length 9-40 bases
- <2°C difference in Tm between the two primers
- maximum of 2 G/C at 3'end.

Probe

- Tm > 10°C higher than primer Tm.
- 20-80% GC content
- length 9-40 bases
- No G on the 5' end
- < 4 contiguous G's
- Must not have more G's than C's

Amplicon

- 50-150 bp in length
- 3' end of primer as close to the probe as possible without overlapping.

2.7 TaqMan Primer titration

Master Mix

Glycerol	60 µl
Buffer A	75 µl
$MgCl_2$	150 µl
dATP	15 µl
dTTP	15 µl
dCTP	15 µl
dTTP	15 µl
AmpliTaq Gold	3.75 µl
UNG	7.5 µl
dH_2O	18.75 µl

Label 10 tubes (1-10) and add 25µl master mix to each. Each tube represents the following concentrations of forward (F) and reverse (R) primers (final concentration in nM):

1	50F:50R	6	300F:900R
2	50F:300R	7	900F:50R
3	50F:900R	8	900F:300R
4	300F:50R	9	900F:900R
5	300F:300R	10	ntc300F:300

Calculate the primer concentration as follows:

$$\text{Volume required} = \frac{0.05(\text{concentration required}) \times 50 \text{ (total volume)}}{\text{Concentration of primer in pmol/ µl}}$$

Add 1 µl probe to all tubes

Add 4 µl cDNA to all tubes

Make volume up to 50 µl with RNase free H_2O.

Aliquot 25 µl to each well in a 96 well TaqMan plate.

Use the following cycling conditions:

50°C x 2 minutes →95°C x 10 minutes →

(95°C x 15 seconds, 60°C x 1 minute) x 40 → refrigerate.

Analyse results and choose the combination which gives the lowest C_T and highest Rn.

2.8 Section 5

2.8.1 Sequencing

To validate the composition of PCR products, TaqMan products and plasmids it was necessary to sequence them. Sequencing was performed using an automated ABI Prism 310 fluorescent sequencer.

2.8.2 Sequencing Chemistry

The dideoxy or enzymatic method, originally developed by F.Sanger (1977), utilises a DNA polymerase to synthesise a complementary copy of a single stranded DNA template. DNA polymerases cannot initiate DNA chains, rather chain elongation occurs at the 3' end of a primer DNA that is annealed to template DNA.. The deoxynucleotide (dNTP) added to the growing primer chain is selected by base-pair matching to the template. Chain growth involves the formation of a phosphodiester bridge between the 3' hydroxyl group at the growing end of the primer and the 5' phosphate group of the incoming deoxynucleotide. Thus overall chain growth is in the 5'→3' direction.

The dideoxy sequencing method capitalises on the ability of DNA polymerases to use analogues such as 2',3'-dideoxy nucleoside triphosphates (ddNTPs) as substrates. When a ddNMP is incorporated at the 3' end of a growing primer chain, chain elongation is terminated at this point due to the lack of a 3'-hydroxyl group. Traditionally, in Sanger dideoxy sequencing, four sequencing ladders are generated with only one of the four possible ddNTPS included in each of four reactions. The ddNTP:dNTP ratio in each reaction is adjusted so that a portion of the elongating primer chains terminates at each occurrence of the base in the template DNA corresponding to the included complementary ddNTP. In this way each of the four elongation reactions contains a population of extended primer chains, all of which have a fixed 5' end determined by the annealed primer and a variable 3' end terminating at a specific ddNTP. Each of the four 'sequencing reactions' contains all four dNTPs, one of which is radiolabelled with ^{35}S, and one of the four ddNTPs. Thus, the reaction product can be visualised by autoradiography following electrophoresis on a high-resolution polyacrylamide gel.

In recent years, there have been a number of advancements in sequencing technology, which have facilitated reproducibility and sample throughput. One such development has been the advent of multicolour fluorescent based sequencing chemistry to replace radioisotope labelling, as used in the instruments and sequencing chemistries provided by PE-Biosystems.

The ABI Prism™ 310 Genetic Analyser is an automated system for sequencing, sizing and quantitating nucleic acids, which integrates ABI Prism™ multicolour fluorescent labelling, capillary electrophoresis and software for data analysis. It employs a laser induced fluorescence, capillary electrophoresis system. The 310 genetic analyser separates DNA fragments using ABI Prism performance optimised polymers (POP) rather than acrylamide, a feature which enhances reproducibility of results. A gel pump automatically loads the sequencing polymer into the capillary, and samples are loaded sequentially by electrokinetic injection. The process is simplified further by fluorescent terminator sequencing chemistry, which allows single-tube sequencing reactions rather than four separate reactions as was previously necessary. Sample detection is achieved by a CCD camera detector which monitors wavelengths from 525-650nm. The optics of the instrument utilises a 10mW argon ion laser for excitation of dyes.

The 310 Genetic Analyser simultaneously detects four different fluorescent dyes in a single capillary, permitting multicolour analysis. Each sample is electrophoresed through the capillary for a specified duration, at which point there is an optically clear segment. At this point laser generated light excites fluorescent dyes as they pass through the 'window'. Each fluorescent dye emits a specific wavelength which is collected in the CCD system. The DNA Sequencing Analysis Software finally processes the data using multicomponent analysis, baseline subtraction and scaling. After processing it detects peaks and determines sequence with 98.5% accuracy up to 425 bases.

2.8.3 310 Gel Pump Assembly

The 310 gel pump assembly fills the capillary with polymer prior to the electrophoresis run. This automatic loading eliminates manual gel pouring.

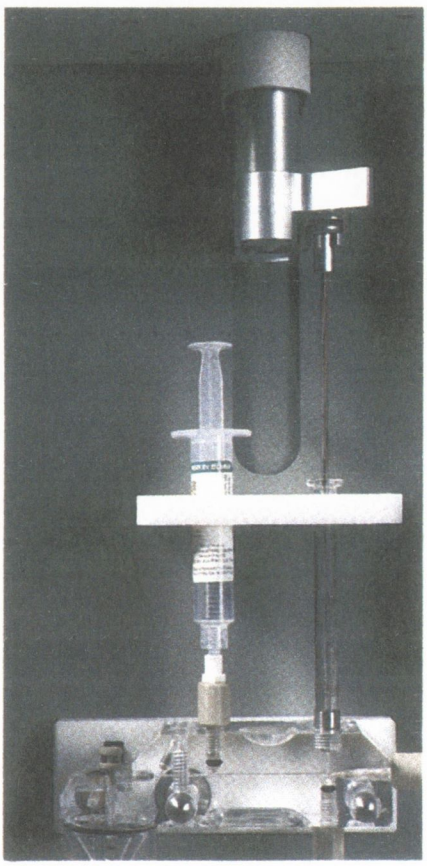

Figure 2.4 Gel Pump Assembly

2.8.4 Sampling System

After samples are placed in the tray, the 310 autosampler loads samples into the capillary before each electrophoresis run. This automated loading ensures that samples are handled consistently and reproducibly.

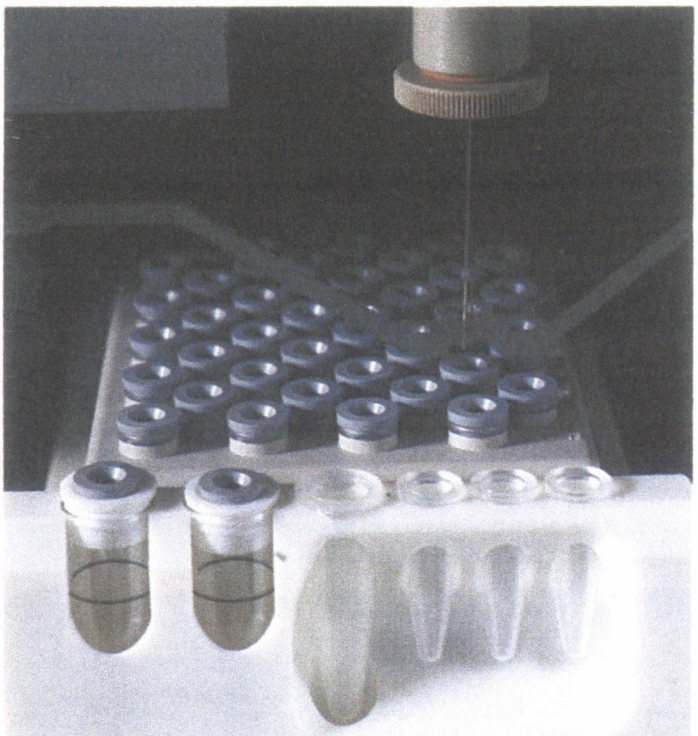

Figure 2.5 310 Auto-sampler Loading System.

ABI Prism BigDye™ Terminators are the most recent development for use in multicolour fluorescent sequencing. This kit employs the sequencing enzyme AmpliTaq DNA Polymerase FS. This variant enzyme has a point mutation at the active site which results in less discrimination against ddNTPs, leading to a more even peak intensity pattern. In addition, the enzyme has a second mutation in the amino terminal domain which virtually eliminates the 5'→3' nuclease activity of AmpliTaq DNA polymerase.

The new dye structures contain a fluorescein donor dye, e.g. 6-carboxyfluoroscein (6-FAM), linked to a dichlororhodamine (dRhodamine) acceptor dye (figure 2.6). The excitation maximum of each dye label is that of the fluorescein donor, and the emission spectrum is that of the dRhodamine acceptor (table 2.1). The donor dye is optimised to absorb the excitation energy of the argon ion laser. The main advantage of the BigDye™ terminators is that they are 2-3 times brighter than standard dye terminators when incorporated into cycle sequencing products. They also have narrower emission spectra giving less spectral overlap and therefore less noise (figure 2.7).

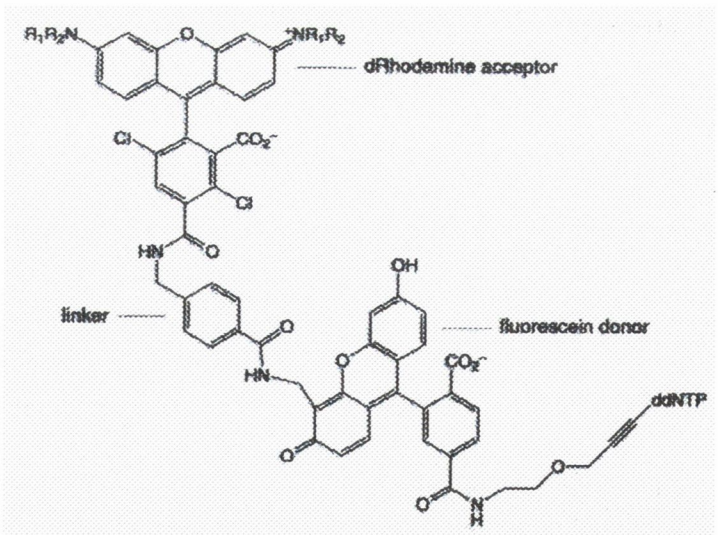

Figure 2.6 General structure of BigDye Terminators.

The BigDye™ terminators are labelled with the following dRhodamine acceptor dyes.

Terminator	Acceptor Dye	Colour of Raw Data on ABI Prism 310 electropherogram
A	dR6G	Green
C	dROX	Red
G	dR110	Blue
T	dTAMRA	Yellow

Table 2.1 BigDye™ terminator labelling.

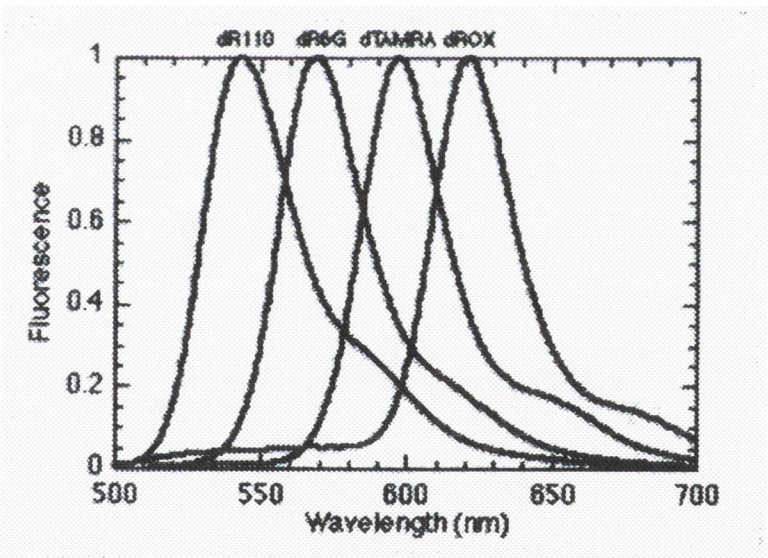

Figure 2.7 Emission spectra of dRhodamine dyes.

2.9 Sequencing PCR products

The PCR product must first be cleaned to remove non-specific products/ left-over primers.

2.9.1 GenElute Agarose Spin Columns

- Run PCR product on a 1.5% agarose gel.
- Cut the desired band from the gel with a sterile scalpel blade.
- Spin Column Wash
    - Add 100µl TE to column.
    - Place in microfuge tube and centrifuge x 5mins @ 10,000rpm.
    - Discard eluent and tube.
- DNA elution and recovery
    - Place the gel slice in the washed column.
    - Centrifuge x10 mins @10,000 rpm.
- Store collected DNA @ -20°C.

2.9.2 Purification of TaqMan products for sequencing.

TaqMan products, by design are considerably smaller than PCR products from conventional solution phase PCR. This poses considerable difficulty in purification of these products, as most 'clean-up' systems remove DNA fragments smaller than 100bp. The following protocol was adopted to purify TaqMan products prior to sequencing:

- Set up a PCR reaction with TaqMan primers for the target sequence required, with the omission of the probe. In addition, the TaqMan 10x buffer (containing ROX) must be replaced by standard 10x buffer.
- Perform the PCR reaction using predefined TaqMan conditions.
- Run the product on a 2.5% agarose gel, stained with ethidium bromide.
- Visualise the product using a UV transilluminator.

- Carefully cut the specific band from the gel (taking care not to include any primer dimmers) and place into a sterile 2.0ml microfuge tube.
- Add 200µl of TAE buffer to the tube.
- Close the tube and place in a horizontal electrophoresis tank containing TAE.
- Apply current, and change the orientation of the tube every 5 minutes to ensure the band migrates from the agarose into the buffer solution.
- Check that the band has completely migrated into the solution, by removing the microfuge tube from the electrophoresis tank and placing it on the transilluminator.
- Remove the agarose slice from the tube and precipitate the DNA as follows:
- Add an equal volume of 4M-ammonium acetate to the extracted PCR product.
- Mix well.
- Precipitate the DNA by adding 2x volume of ice-cold ethanol and place at – 70°C x 30 minutes.
- Spin x 5 minutes at maximum speed in a benchtop centrifuge.
- Remove the supernatant and wash the 'pellet' with 1ml of 70% ethanol.
- Mix, invert tube several times and spin x 5 minutes.
- Remove supernatant and dry the pellet under vacuum.
- Resuspend the DNA in 20µl of sterile dH_2O.

2.9.3 Cycle Sequencing

- Use ~ 8µl of purified PCR product
- Primers – require 7.5pmol/20µl reaction.
- Add 8µl of ready reaction mix.
- Use the following thermal cycling parameters:

[96°C x 10secs →50°C x 5 secs → 60°C x 2 mins] x 25.

2.10 Section 6

2.10.1 Cloning

TOPO™ TA Cloning provides a highly efficient, fast, one-step cloning strategy for the direct insertion of Taq polymerase-amplified PCR products into a plasmid vector. The plasmid vector (pCR 2.1-TOPO™) is supplied linearised with single 3' thymidine (T) overhangs for TA cloning, and Topoisomerase covalently bound to the vector ('activated' vector).

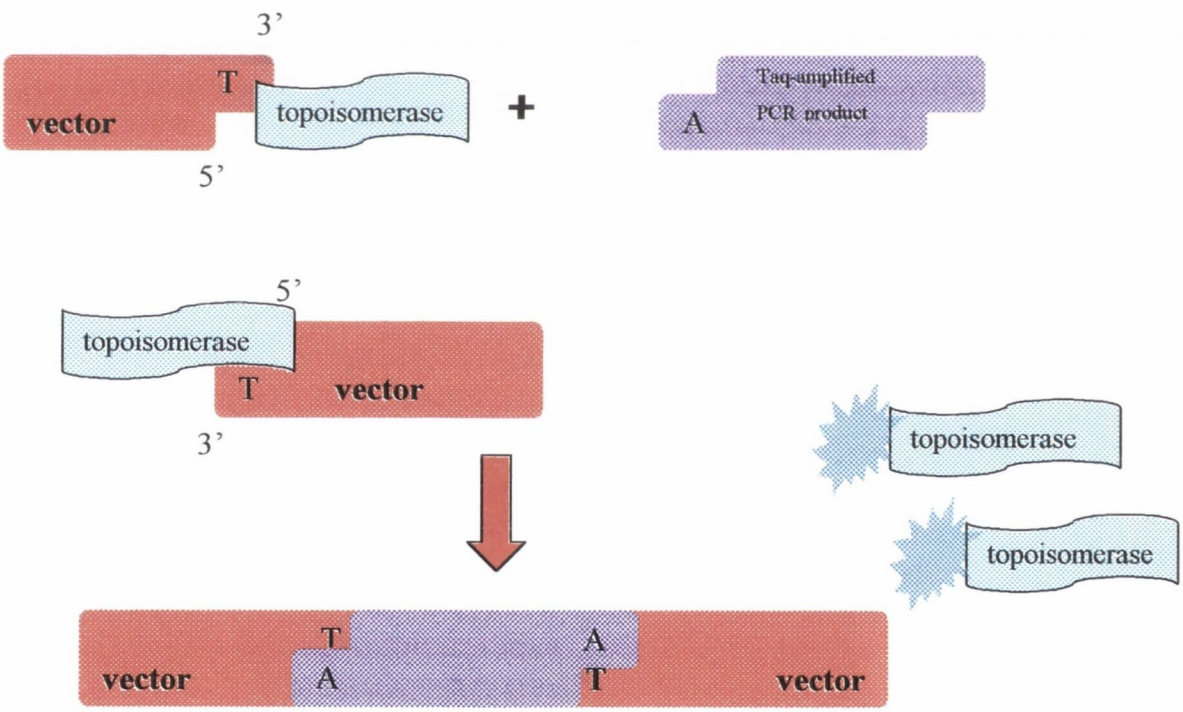

Figure 2.8 TOPO™ TA Cloning Reaction

Taq polymerase has a non-template dependent terminal transferase activity which adds a single deoxyadenosine (A) on to the 3' ends of PCR products. The linearised vector has single, overhanging 3' deoxythymidine (T) residues. This allows the PCR inserts to ligate efficiently with the vector.

TOPO™ Cloning exploits the ligation activity of topoisomerase by providing an 'activated', linearised TA vector. Ligation of the vector with a PCR product containing 3' A overhangs is very efficient and occurs spontaneously within 5 minutes at RT°C. The TOPO™ Cloning reaction can be transformed into chemically competent cells.

2.10.2 Ethanol Precipitation of Sequencing Product

- Transfer 20µl of sequencing reaction to 1.5ml microfuge tube.
- Add 80µl 75% Isopropanol.
- Close the lid, vortex and leave at RT°C x 15 mins.
- Centrifuge x 20 mins @ max rpm.
- Immediately remove all supernatant and discard.
- Add 250µl 75% Isopropanol.
- Vortex and centrifuge x 5 mins.
- Remove supernatant and discard.
- Dry samples under vacuum.
- Add 25µl template suppression reagent (TSR) to tubes.
- Mix and vortex.
- Incubate @ 95°C x 2 mins to denature samples.
- Immediately place on ice.

2.10.3 Cloning Reaction using Invitrogen TOPO ™ TA Cloning Kit.

- Set up PCR reactions.
- Place LB plates @ 37°C.
- Add 40µl X-Gal and 40µl IPTG to each plate.
- Thaw SOC medium.
- Thaw 1 vial of cells per reaction. Note: do not start the cloning reaction until the cells are thawed. Allow the cells to thaw slowly on ice and do not shake or mix them.
- Gel purify PCR products using GenElute columns.

2.10.4 Cloning Reaction:

- 2µl PCR product
- 2µl dH_2O
- 1µl vector (Topo 2.1)
- Mix gently
- Incubate at RT°C x 5 mins.
- Briefly centrifuge

2.10.5 Transformation Reaction

- To each tube of cells add:
  - 2µl βME (beta-mercaptoethanol)
  - 2µl above cloning reaction
- Leave on ice x 30 mins.
- Heat-shock cells @ 42°C x 30 secs.
- Place on ice x 2 mins.
- Add 250µl SOC medium to each tube.
- Place @37°C x 2 hours.
- Plate 50/100ml onto prepared LB plates.
- Incubate @37°C overnight.

2.11 Section 7

2.11.1 Sequences obtained from plasmids, PCR products and TaqMan products.

2.11.2 TaqMan products for TSHr and *ret*/PTC-1

The products were obtained and purified as described p56, and cycle sequencing was performed using BigDye terminators.

As the amplicon size for TaqMan products is small, it was not possible to get the entire sequence from single direction sequencing due to the inevitable primer flare at the beginning of the readout form the 310 analyser. However, by combining the readings from both forward and reverse strands the sequence of the full transcript could be validated.

2.11.3 TSHr Sequence

TSHr sequence: CACAC GGGCT GACCT TTCTT ACCCA AGCCA

Forward primer: ……..CA

Reverse primer: GTGTG CCCGA CTGGA AAGAA TGGGT TCGGT

TSHr sequence: CTGCT GTGCC TTTAA GAATC AGAAG

Forward primer: CTGCT GTGCC TTAA GAATC AGAAG

Reverse primer: GACGA CACGG AATT CT……..

TSHr sequence: AAAAT CAGAG GAATC CTTG

Forward primer: AAAAT CAGAG GAATC CTTG….

Reverse primer:

The text in black represents the sequence of the TSHr TaqMan transcript. The blue text represents the sequence obtained from sequencing with the forward primer, while the red is the output generated from the reverse primer.

2.11.4 *ret*/PTC-1 sequence

ret/PTC/1 sequence: CGCGA CCTGC GCAAA GCCGG CGTTA CCATC

Forward primer: ……TA CCATC

Reverse primer: GCGCT GGACG CGTTT CGGCC GCAAT GGTAG

---

ret/PTC/1 sequence: GGGGG TCCGG GGTGG GAATT CCCTC GGAAG

Forward primer: GGGGG TCCGG GGTGG GAATT CCCTC GGAAG

Reverse primer: CCCCC AGGCC CCACC CT……

---

ret/PTC/1 sequence: AACTT G

Forward primer: AACTT G

Reverse primer: ……

The sequences described here follow the same colour code as TSHr on the previous page.

2.11.5 Sequence of TSHr plasmid.

This plasmid was generated to serve as a control for TaqMan quantitation assays. Having generated the plasmid, it was necessary to validate the sequence. BigDye terminators were used for cycle sequencing with the ABI 310 autoanalyser.

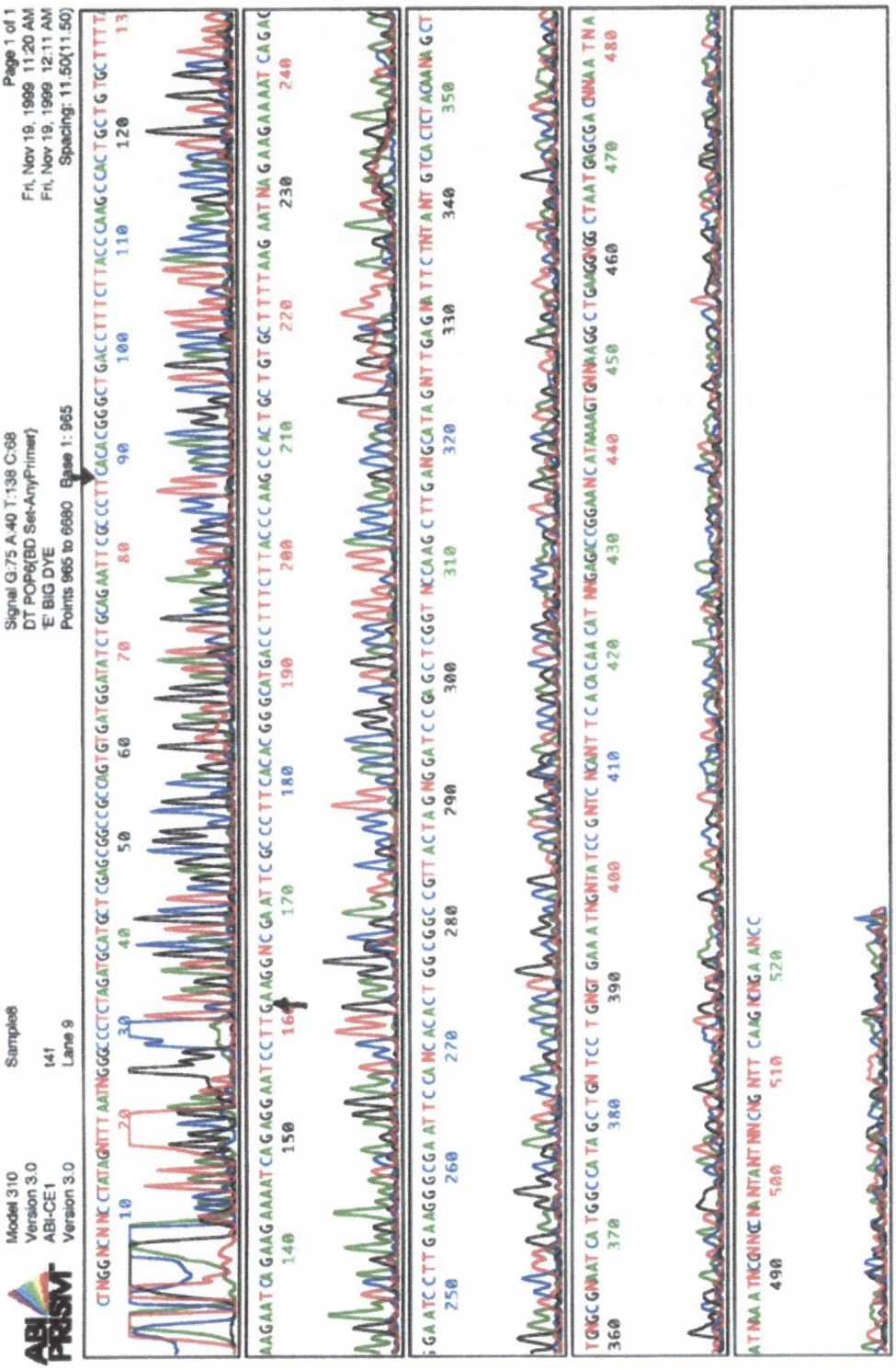

Figure 2.9 Sequence of TSHr from cloned plasmid.

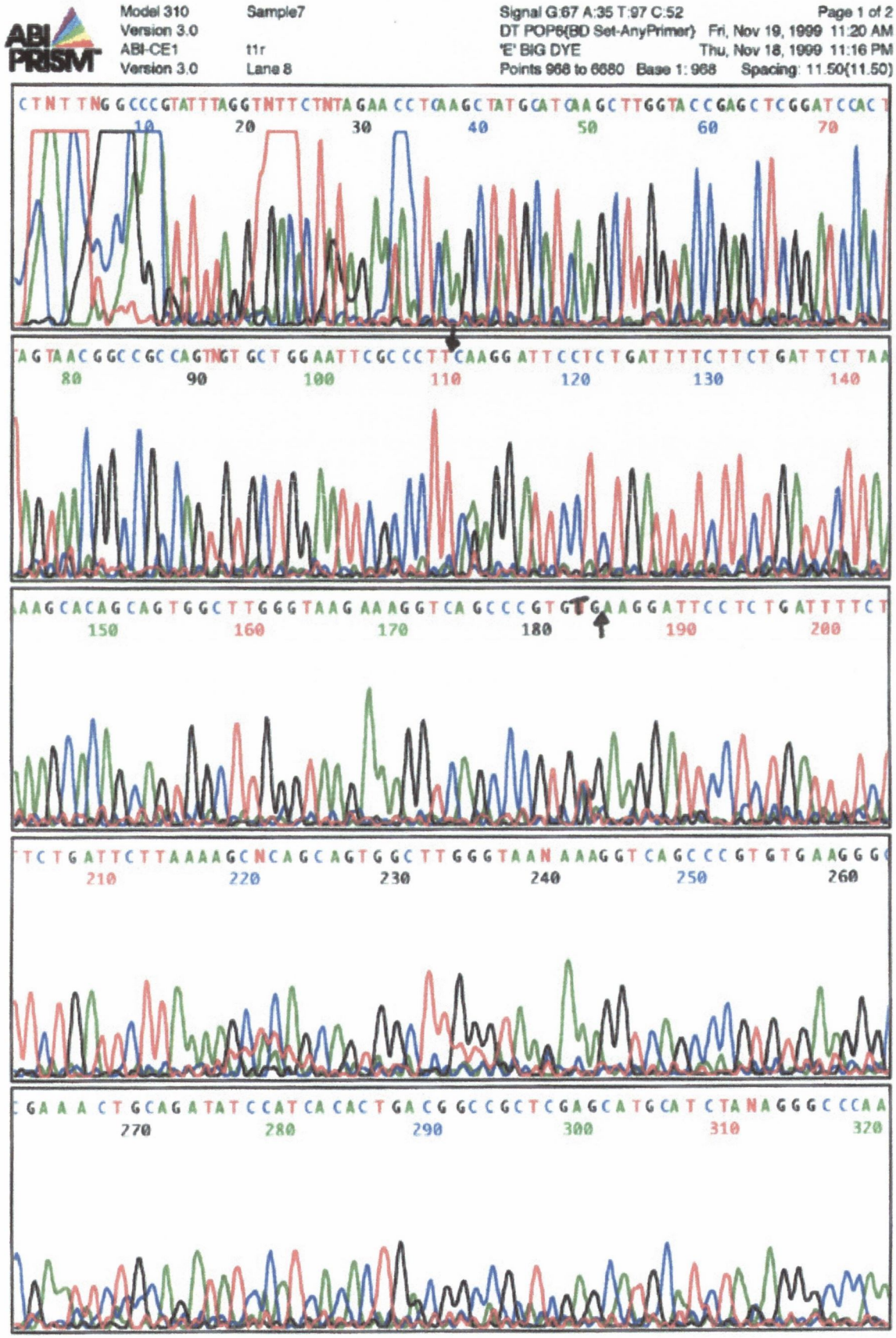

Figure 2.10 Sequence from reverse primer for cloned TSHr plasmid.

A pGEM control plasmid (with a known sequence) was sequenced as a control to ensure the sequencer was operating accurately. The derived sequence was as follows:

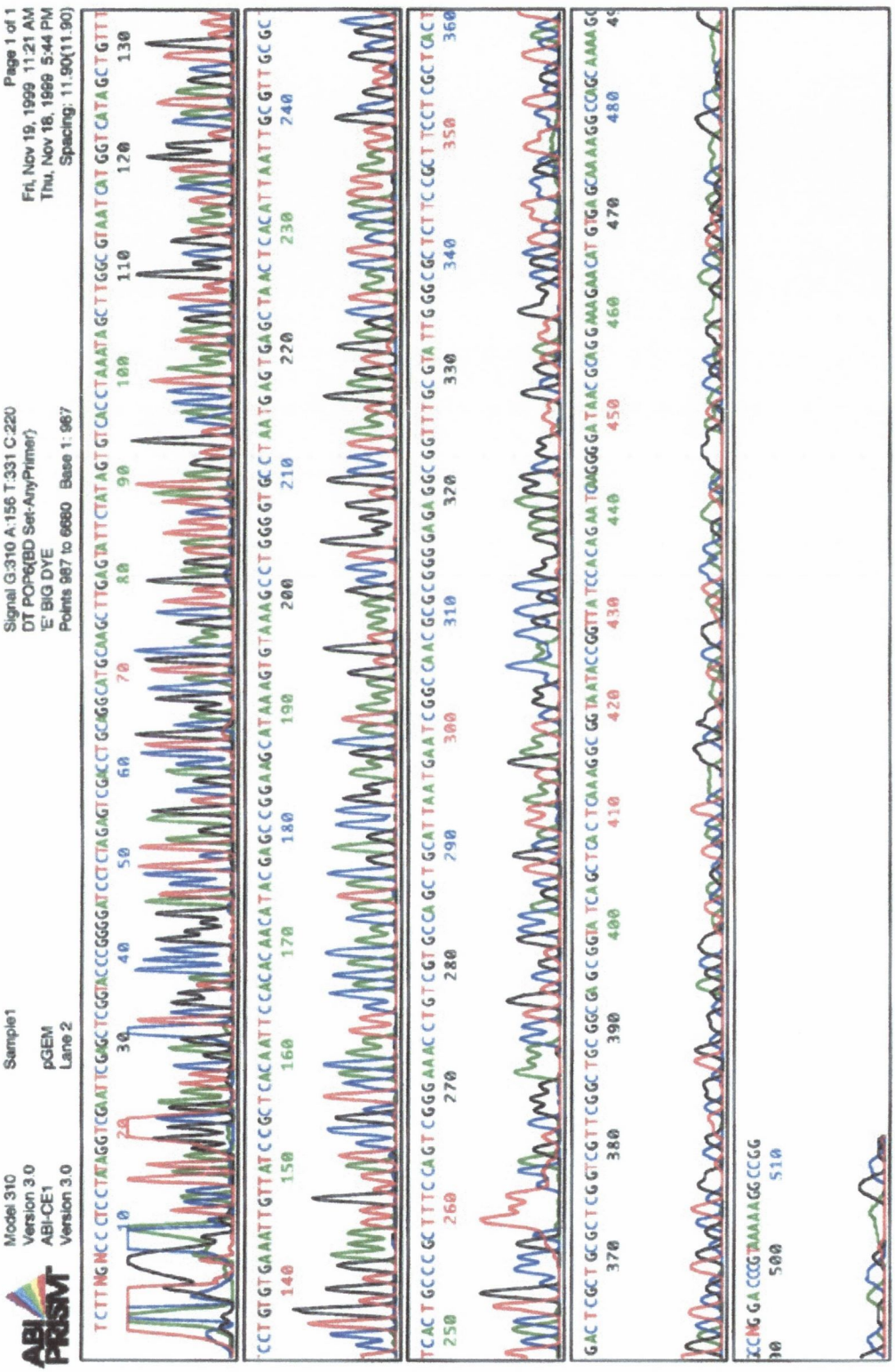

Figure 2.11 Sequence from pGEM control plasmid

A set of outer primers were generated external to the TaqMan sequence for TSHr for use in the generation of a plasmid for quantitation purposes. The validated sequence derived from these primers is as follows:

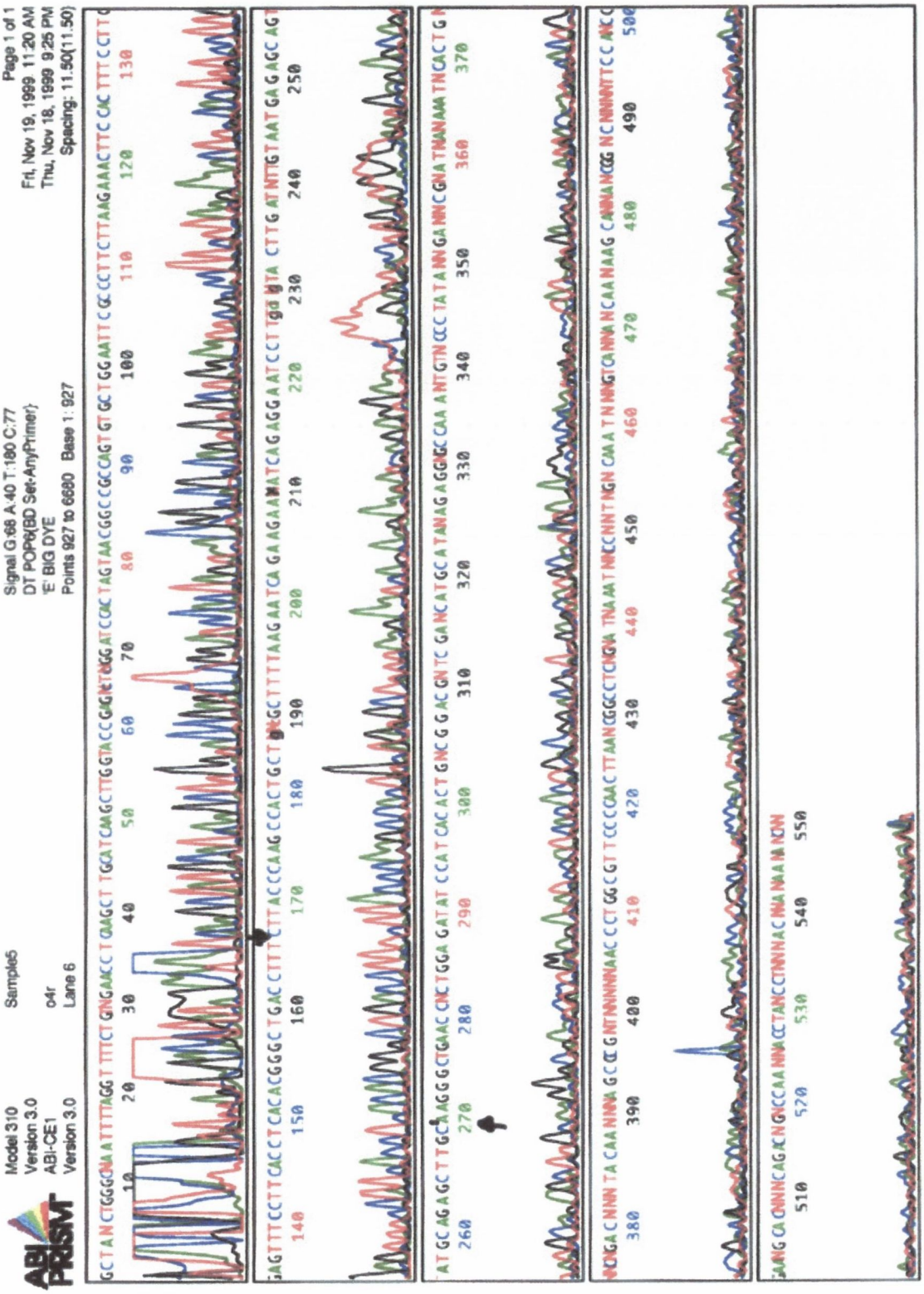

Figure 2.12 Sequence of external TSHr product.

2.12 Section 8

2.12.1 *In-situ* TaqMan RT-PCR

The localisation of genes in specific cells and tissues has been instrumental in many disciplines for defining disease pathogenesis. A major limitation of solution-phase PCR is the inability to visualise and localise amplified product within cellular and tissue specimens. *In situ* hybridisation (ISH) enables specific nucleotide sequences to be localised at the cellular level. *In situ* PCR or RT-PCR represents the union of two separate techniques: PCR or RT-PCR and ISH. *In situ* TaqMan RT-PCR merely advances this concept a step further.

In this study, TaqMan RT-PCR was applied to the TPC-1 cell line. This cell line is derived from a papillary thyroid carcinoma (PTC) known to harbour the *ret*/PTC-1 chimeric transcript. The study was undertaken to identify the localisation of the transcript in the cultured PTC cells.

2.12.2 rTth enzyme

This enzyme can be used to avoid the complications of a two stage reverse-transcription/cDNA amplification procedure. The method involves the use of a single buffer to carry out both reverse transcription and the PCR amplification steps using a single enzyme recombinant Thermus thermophilus (rTth) DNA polymerase. Using a bicene buffer system and $Mn(OAc)_2$, the rTth enzyme acts both as an RNA and a DNA dependent DNA polymerase, thus avoiding the problems of a two step approach. Furthermore, rTth is a highly thermostable polymerase that enables reverse transcription to be carried out at temperatures where standard mesophilic enzymes would be inactive. Performing the RT step at high temperatures ensures high specificity, reduced background and increased signal-to-noise ratios.

2.12.3 Fixation

Incubation in detergent solutions produces sufficient permeabilisation of cultured cells to permit *IS*-PCR. Using Permeafix (Ortho Diagnostics) cells may be simultaneously fixed and permeabilised prior to *in situ* amplification by RT-TaqMan.

2.12.4 Controls

As with all techniques it is imperative to include adequate controls with IS-TaqMan RT- PCR. Such controls include omission of the probe, primers, rTth enzyme. A Manganese titration should also be performed for each assay type to optimise its concentration. It is also important to check a slide that has not been subjected to thermal cycling but has had all the reaction components applied.

2.12.5 Slide assembly

The IS PCR 1000 system (PE-Biosystems) comes with a slide assembly tool. In order to provide a liquid tight seal of RT-PCR reagents over the sample on the slide, a two part containment system is used. This comprises the slide and stainless steel Amplicover Clips with silicone rubber Amplicover Discs.

The slide is placed on the pre-heated assembly platen. The RT-PCR reaction mix is applied over the cellular area. The AmpliCover Clip with the AmpliCover Disc inserted are placed in the handle of the assembly tool. They are held in place by a magnet. The handle is drawn down over the slide and a concentric cam operated by the handle engages with the lid and draws the AmpliCover assembly onto the surface of the slide, forcing out air and creating a liquid tight seal. Two push-operated levers on the upper surface of the lid the stainless steel clips onto the slide and create a reaction containment system. After cycling the clips are removed and may be re-used.

2.12.6 Protocol

- Culture TPC-1 cells to confluence
- Trypsinise cells
- Aliquot cell suspension and make cytospin preparations onto APES coated slides specially prepared for IS-PCR (PE-Biosystems).
- Allow slides to air dry.
- Fix slides in 1x Ortho Permeafix x 90 mins.
- Place slides in 40% Ethanol x 30 mins.
- Place slides in 60% Ethanol x 30 mins.
- Place slides in 80% Ethanol x 30 mins.
- Place slides in 100% Ethanol x 30 mins.
- Store slides in 100% Ethanol @ -70°C until ready for staining.
- Label slides as follows:
  - 1 = 2.5mM Mn^{2+}
  - 2 = 3.5mM Mn^{2+}
  - 3 = 4.5mM Mn^{2+}
  - 4 = no probe
  - 5 = no rTth
  - 6 = no primers
  - 7 = 0 cycles
- Assemble slides and reagents.
- Perform 25 cycles using a PE in-situ PCR 1000 thermocycler using the following reaction conditions:
  - 58°C x 45 mins
  - 94°C x 5 mins
  - [94°C x 45 secs, 60°C x 1'30"] x 25
- Wash slides in 2x SSC (Sigma S-6639 = 20x stock) @ 37°C x 10 mins
- Rinse slides in DEPC water.
- Mount in 25µl Vectashield mounting medium with DAPI (diaminophenylindone).
- Visualise slides using Leica DXM-RXA microscope and Q-Fish software

2.12.7 IS-TaqMan RT-PCR Master Mix

Master Mix x 6 samples

Volume	Component	Final Concentration
60µl	5x EZ buffer	1x
48 µl	25mM dNTP's	0.4mM
12 µl	Forward Primer	10µM
12 µl	Reverse Primer	10µM
9 µl	TaqMan Probe	5µM
42 µl	Mn^{2+}	3.5mM
99 µl	RNase free H_2O	
18 µl	rTth	75U

Stock Mn^{2+} and RNase free H_2O volumes should be adjusted as follows for titration (per sample):

Concentration→	2.5mM	3.5mM	4.5mM
Mn^{2+}	5 µl	7 µl	9 µl
H_2O	18.5 µl	16.5 µl	14.5 µl

2.13 References

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CHAPTER 3

ANAPLASTIC THYROID CARCINOMA: CELL KINETICS, p53 EXPRESSION AND SYNTHETIC FUNCTION.

3.1 SUMMARY:

The focus of this chapter was to evaluate a panel of tumour markers in a pilot group of thyroid tumours, and to determine their prognostic value. Proliferation indices (Ki-67, EGFr and mitotic count), oncogene expression (p53), thyroglobulin synthesis, TSH receptor expression and apoptotic rate were assessed in 8 cases of anaplastic thyroid carcinoma (ATC) and the results compared with those from 12 cases of various differentiated thyroid epithelial neoplasms (TEN). Mitotic figures, apoptotic and immunohistochemically reactive cells were counted per 1000 tumour cells in consecutive fields.

Anaplastic thyroid carcinoma (ATC) is an uncommon, aggressive malignancy constituting 12% of a series of 80 thyroid carcinomas collected from archives over a ten year period. While proliferative activity and oncogene expression in various other forms of thyroid carcinoma have been studied extensively, little work has been done to correlate these parameters or to assess the modifying effect of apoptosis on tumour growth in ATC.

Median counts for Ki-67 positive cells and mitotic figures were 29.9% and 1.1% respectively in ATC, compared with 1.5% and 0.13% in the control group. Apoptotic counts in ATC yielded a median of 1.43% compared with 0.1% in controls. Significant expression of EGFr and p53 was identified in 5 and 6 ATC respectively, whereas only 2 control cases showed significant staining for one or both of these markers. Levels of p53 expression were found to be inversely related to thyroglobulin staining. Relative TSH receptor (TSHr) expression detected by RT-PCR showed a marked decrease in undifferentiated tumours.

3.2 Introduction

3.2.1 Classification of thyroid tumours

The framework of the classification of thyroid tumours has remained relatively stable in the 30 years since Hedinger and Sobin (1974) co-ordinated the first edition of the W.H.O. booklet on *Histological Typing of Thyroid Tumours*. Such stability is generated from the concept that thyroid carcinomas originate from the two main types of thyroid cells (follicular and C-cells), and can be divided into four main categories of tumours: follicular, papillary and undifferentiated (anaplastic) carcinomas (derived from follicular cells), medullary carcinomas (derived from C-cells).

During the 1980s, reports of thyroid tumours which failed to fit comfortably into the aforementioned categories began to emerge. For example, Carcangiu et al (1984) identified a group of tumours, which fell somewhere between differentiated and undifferentiated carcinomas, which displayed a prominent insular pattern. Such observations prompted the publication of the 2nd edition of *Histological Typing of Thyroid Tumours* by W.H.O (1988). The criteria advanced by Hedinger, LiVolsi (1990) and Rosai (1992) were used in a review of a large series of thyroid tumours which had been studied by routine histopathological methods, immunocytochemistry, electron microscopy and in-situ hybridisation. This review lead to the revised classification of primary thyroid carcinomas.

3.2.2 Prognosis

The prognosis of differentiated thyroid carcinoma is generally good, but because 10-20% of patients demonstrate significant progression of their disease, substantial efforts have been made to pinpoint those patients whose disease will advance. Patients in this group are candidates for more aggressive treatments based on individual prognostic factors, such as age, distant metastasis at diagnosis, primary tumour size, gender and histologic grade. In addition, certain pathological features such as marked nuclear atypia, necrosis or vascular invasion are also important.

In this study, a series of potential prognostic indicators were examined in a cohort of thyroid neoplasms, and the results compared with histological grade.

3.2.3 Proliferation

The assessment of cell proliferation using a variety of techniques remains popular in laboratories worldwide. This activity is promoted by the belief that measuring the parameters of cell proliferation will provide objective information about tumours which will allow more effective management of patients. Antibodies to the Ki-67 antigen can now be applied to formalin fixed, paraffin embedded archival material (Cattoretti 1992) and the use of such reagents is an effective measure of the growth fraction of a cell population. The antibody detects an antigen present in all stages of the cell cycle except G_0 (Gerdes 1983,1984).

The remarkable conservation of the central mechanisms of cell cycle control has been elucidated over the last decade. It is clear that there is a highly co-ordinated

set of inter-dependent biochemical reactions which regulate cell cycle progression. Many of these involve cascades of phosphorylation and dephosphorylation of regulatory proteins, which ultimately leads to highly organised structural events, such as DNA synthesis in S phase and the process of mitosis.

There are a number of distinct aspects central to the concept of cell proliferation. The $p34^{cdc2}$ protein is a key regulator of cell cycle kinetics and is involved in the transition between G_2 and mitosis. It is a protein kinase which acts in combination with one of the members of the cyclin family of proteins. Another group of protein kinases cdc2-like kinases are collectively termed cyclin dependent kinases (CDKs). They act in collaboration with cyclin and are involved in the regulation of the cell cycle. Finally, the recognition of mechanisms that act as checkpoint controls for cell cycle progression such as p53 are inextricably linked to the control of cell cycle kinetics.

3.2.4 Growth Factors

The epidermal growth factor receptor (EGFR) is a member of the protein tyrosine kinase superfamily of receptors, encoded by the *c-erbB* proto-oncogene. The EGFR gene can be activated to a transforming oncogene by mutational events, leading to a structurally and functionally altered gene product, and/or by an increase in gene dosage through gene amplification. EGFR is a potent mitogen for thyroid follicular cells in culture (Duh 1985). The mitogenic effect of EGFR can be modulated by thyrotropin (TSH) and by an increase in the number of EGFR molecules expressed by the follicular cell (Kasai 1987).

3.2.5 Checkpoint controls in the cell cycle –p53

Since its discovery almost 20 years ago, the p53 gene and its protein products have been the subjects of an almost unprecedented investigation. Justification for this lies in the critical role p53 plays as one of the cell cycle checkpoints that control proliferation and differentiation (Lane 1994). p53 is a tumour-suppresssor gene found on chromosome 17p, and alterations (missense mutation or deletion) within the coding sequences of the gene are among the most frequent genetic changes detected in human neoplasia. Point mutations in the p53 gene result in increased stability of the mutant protein, which can be detected by immunohistochemistry, whereas the wild-type p53 protein is generally undetectable due to its short half-life

3.2.6 Apoptosis

The concept of cell-cycle checkpoints reminds us of the importance of the regulation of growth-arrest. Much of the literature on assessing the rate of growth of tumours has been based on the premise that positive regulation of the cell cycle is the main control mechanism. However, examination of normal adult tissues indicates that most cells are not dividing. The quiescent population is highly organised and regulated. This regulation must be active and not passive (Kerr 1972, Raff 1992). There is now evidence that specific genes are expressed in association with growth arrest states. These control mechanisms are very important, since even a small alteration in the amount of cell division in a population will radically alter the population size. Another component of the control of cell number in a tissue is the rate of cell loss. Recently, the phenomenon of apoptosis, and its control has been the focus of large numbers of publications

(Staunton et al 1998). Genes such as *bcl*-2, *c-myc* and *p53* are intricately involved in this process, and their role is being further elucidated as the molecular mechanisms of apoptosis are discovered.

There are a variety of techniques available for assessing apoptosis, ranging from detection of apoptotic bodies on H&E stained sections, to in-situ end-labelling of DNA fragments in apoptotic cells, to electron microscopy. The duration of the apoptotic process is short and most estimates put the clearance time at less than one hour. Consequently, even small apoptotic body counts may account for large cell fluxes. The assessment of the number of apoptotic bodies may be an essential parameter in predicting the clinical behaviour of a given tumour.

Such considerations are also true of mitoses, which account for only a small component of the cell cycle. Moreover, the duration of mitosis is variable, particularly in neoplasia.

The data obtained by assaying cell proliferation, growth arrest or cell death is of little value if taken in isolation. However, comparisons with known prognostic factors or clinical data such as disease free survival are of importance.

This study evaluates proliferation indices (Ki-67, EGFr and mitotic count), oncogene expression (p53), TSHr expression and apoptotic rate in 8 cases of ATC (5 female, 3 male, average age at diagnosis 69yrs) and compares the results with those derived from a group of other thyroid neoplasms (comprising papillary (n=5), follicular (n=3) and medullary (n=2) carcinomas and follicular adenomas (n=2), 7 female, 5 male, average age at diagnosis 48yrs) used as controls.

Ki-67 positive cells and mitoses were counted per 1000 tumour cells in consecutive fields. The percentage median count for each of these parameters in ATC was 29.9% and 1.1% respectively, compared with 1.5% and 0.13% in the control group.

Significant expression of EGFr and p53 was identified in 5 and 6 of ATC respectively, whereas only one for each marker of the control cases showed significant staining. The level of p53 expression was inversely correlated with immunohistochemical evidence of thyroglobulin synthesis in ATC. Apoptotic counts per 1000 cells in ATC yielded a median of 1.43% compared to 0.1% in controls.

Comparison of the intensity of bands obtained by RT-PCR for an internal control (Abl) to those for TSHr was used to assess relative TSHr expression. The ratio of TSHr:Abl detected was found to be markedly reduced in ATC (0.29), compared with the control cohort (0.88), indicating decreased expression of TSHr. This form of semi-quantitative assessment while crude, highlights the potential of PCR based technology in yielding valuable information with respect to functional genomics, and will be developed further in the following chapters.

In conclusion, the greater proliferative activity of ATC, compared to other forms of thyroid neoplasia, is matched by greater p53 and EGFr expression, a correspondingly higher apoptotic rate and a decrease in TSHr expression.

3.3 Materials and Methods

10 cases of ATC were obtained from archival material accessioned over a ten year period. Of these 10 cases, 8 had sufficient material for study. Five patients were male and three female with average age of 69 years (range 58-87). The histological diagnosis was made according to recognised criteria (AFIP), on haematoxylin and eosin (H&E) stained slides with immunohistochemistry as an adjunct. All tissues had been fixed in 10% buffered formalin, and paraffin embedded. The ATC were compared with a series of controls comprising: 3 Follicular carcinomas (FTC), 5 Papillary carcinomas (PTC), 2 Medullary carcinomas (MTC) and 2 Follicular adenomas (FA) from 7 female and 5 male patients with average age 48 years (range 24-69).

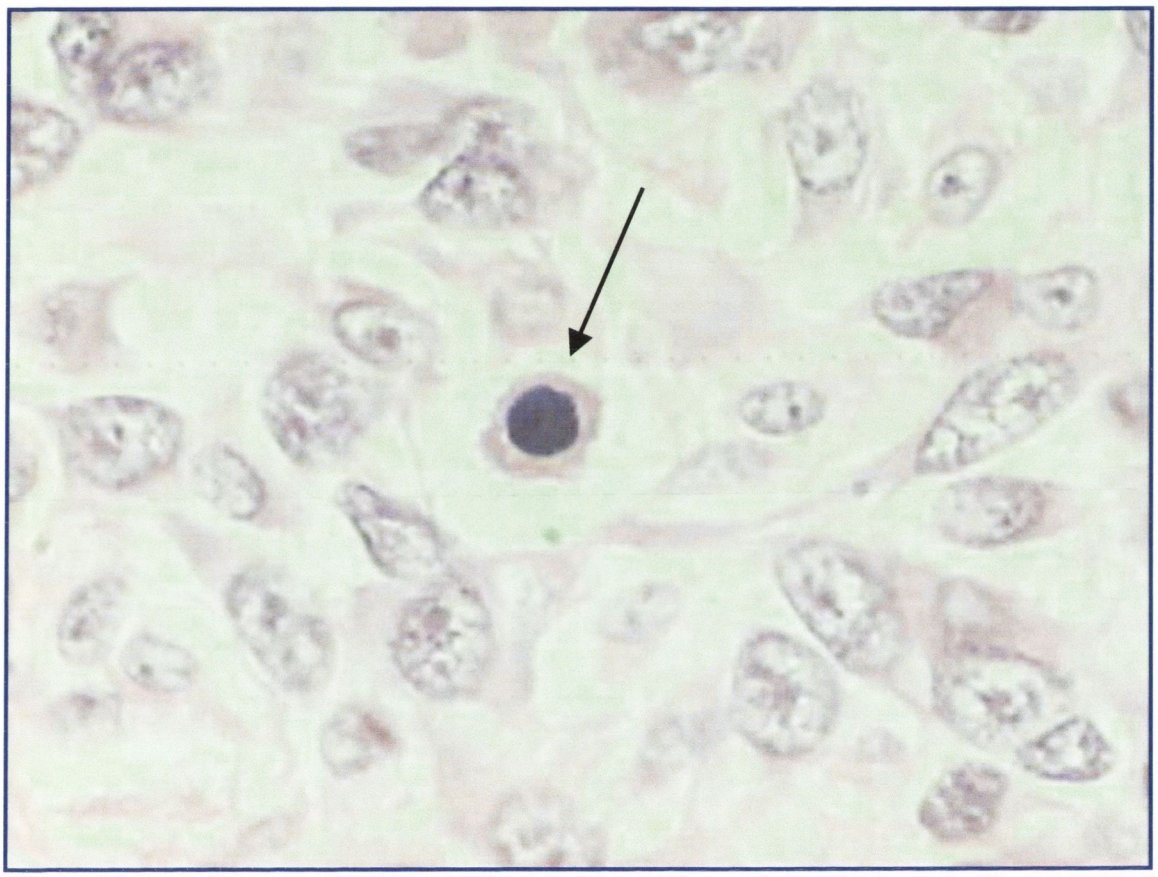
3.3.1 Mitotic and Apoptotic Counting.

Mitotic and apoptotic bodies were counted on 4μm H&E sections as a percentage of 1,000 consecutive tumour cells at 400× magnification. Apoptotic bodies were identified as cells with characteristic cytoplasmic and nuclear condensation (figure 3.1).

Figure 3.1

Apoptotic cell (arrowed) in an anaplastic thyroid carcinoma.

H&E Stain, x400 magnification.



3.3.2 Immunohistochemical Staining

4μm sections were cut onto APES coated slides (3-aminopropyltriethoxysilane [Sigma Chemical Co., St. Louis, MO]) and stained by the avidin-biotin-peroxidase complex (ABC) method (Vector Labs, Burlingame, Ca.), for Ki-67, thyroglobulin, calcitonin and CEA (all DAKO, Glostrup, Denmark), p53 DO7 (Novacastra, UK) and EGFr (Triton Diagnostics, Alameda Ca).

For both Ki-67 and p53 staining, microwave antigen unmasking in 0.01M Citrate buffer (pH 6.0) was used.

Staining which was sufficiently contrasted as to show up on a black / white photograph was taken as the threshold for positivity. Known positive and negative slides were run as controls in each batch of staining. All counts were performed by a minimum of two people and there was no significant inter-observer variation.
Ki-67, EGFr and p53 positive cells [figure 3.2], as in the case of mitoses and apoptotic bodies were counted as a percentage of 1000 consecutive tumour cells at 400× magnification.

3.3.3 RT-PCR

2 x 20μm sections were cut into DEPC treated sterile 1.5ml microfuge tubes. Total RNA was extracted using the described modifications to the Purescript kit (Gentra Systems Inc., MN) protocol. Reverse transcription and subsequent PCR were performed using a GeneAmp RNA PCR core kit (Perkin Elmer, Foster City CA) on a PE480 thermocycler.
To confirm the integrity of the RNA, and to act as a comparative baseline ABL RNA transcripts were chosen as a reporter target using primers from exons 1a ,1b and 2 (Roth et al 1989).

The primers used for TSHr gave a 118bp product, which encompassed exons 7 to 9 (bases 642-759).

Amplicons were electrophoresed on low melting point agarose gels (figures 3.3, 3.4). Bands were visualised using a UVP 7500 gel documentation system and analysed by densitometry using a Pharmacia LKB-image Master with 'Diversity One' software.

Immunohistochemistry for Abl expression in both benign and neoplastic thyroid tissues did not demonstrate any differences in staining pattern or intensity, validating its use as a housekeeping gene with this tissue type.

Tissue blocks were cut and analysed in duplicate to confirm consistency of results. The intensity and area occupied by each band in each lane was computed using densitometry and reported as a percentage of the total for each lane. The percentages obtained for TSHr and Abl were divided, and this ratio was used as an index of TSHr expression.

3.4 RESULTS

The growth fraction in ATC as estimated by Ki-67 staining (median =29.9%) was greater than in any other form of thyroid epithelial neoplasm (median = 1.5%).

Receptors for epidermal growth factor were more frequently detected (6/8), and in greater concentration (median = 49%), than the control group (1/12, median= 8%).

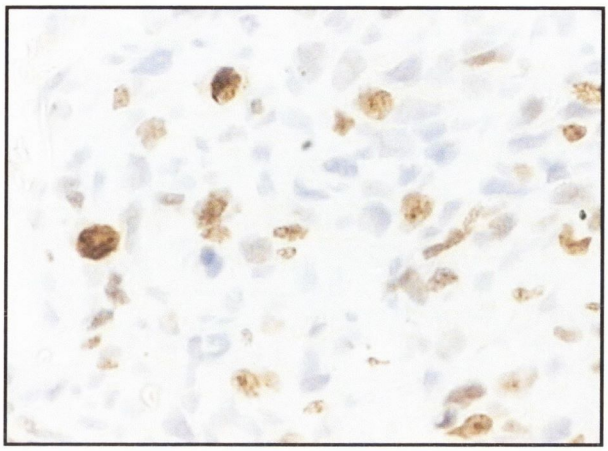
Similarly p53 staining was detected in all 8 ATC (median = 44.6%), while 2/12 of the control group showed positivity (median = 0.05%).

Demonstration of EGFr and p53 were inversely related to thyroglobulin staining probably indicating a degree of dedifferentiation. Normally growth and synthetic activity go hand-in-hand (as in the case of Grave's Disease), but they become uncoupled in the process of mutation.

The median ratio of growth fraction to apoptosis (median-Ki-67 : median-apoptosis) in ATC was 21.4 compared with 1.5 in the remaining TENs.

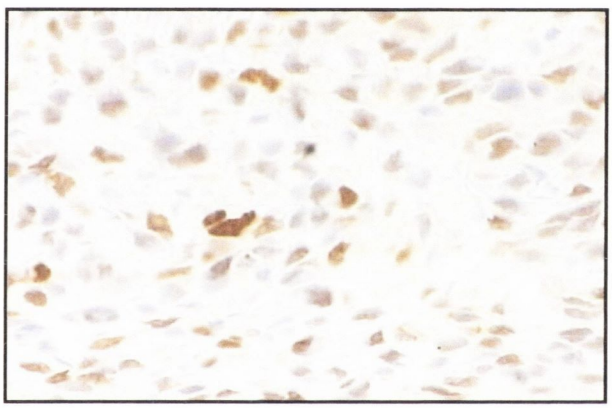
The calculated TSHr index showed decreased expression of TSHr in ATC (median = 0.29) compared with the control group (median = 0.88).

A summary of these results is displayed in table 3.1



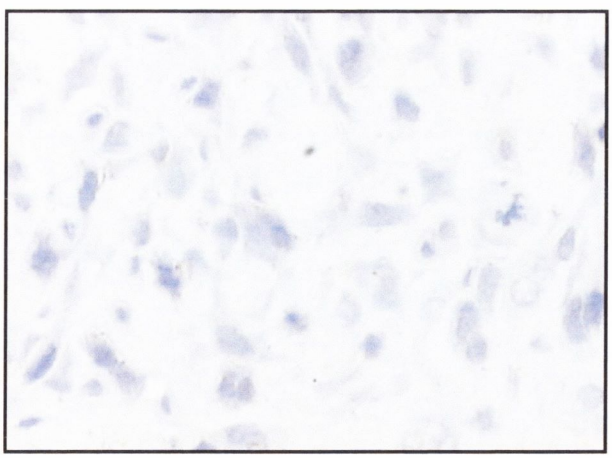
p53 (DO7) immunostaining. Brown nuclear staining is present in positive cells

(Counterstain = Mayer's Haematoxylin)



Ki-67 immunostaining in ATC case. Positive staining is localised to the nucleus. Note the variation of staining intensity among different cells.

(Counterstain = Mayer's Haematoxylin)



Immunostaining for thyroglobulin is negative in this undifferentiated ATC.

(Counterstain =Mayer's Haematoxylin)

Figure 3.2 Immunohistochemistry for p53, Ki-67 and thryoglobulin on an ATC.

Case	Type	Rel. Abl:TSHr	Ki-67	EGFr	Mitosis	Apoptosis	p53	TGN
1 m 76	ATC	0.44	22	0	1.6	3.8	98	-
2 f 66	ATC	0	20	0.5	0.3	0.8	45	-
3 f 64	ATC	0	31	98	0.8	0.6	1.6	+
4 f 74	ATC	0.57	35	0	0.4	2.6	0.2	+
5 m 77	ATC	0.30	25	90	0.4	1.8	79	-
6 f 87	ATC*	0.39	25	13.4	1.8	1.1	0.2	+
7 f 58	ATC	0.59	28	92	0.8	0.4	45	-
8 m 63	ATC	0	53	98	2.8	0.3	88	-
9 f 52	FTC	0.90	1.5	0	0	0.4	0	+
10 f 24	FTC	0.82	0.7	0	0.1	0.1	0	+
11 m59	MTC	0.94	0.2	0	0.2	0.1	0	-
12 m26	MTC	0.77	1.3	96	0.5	0.3	0.3	-
13 f61	PTC	0.82	4.1	0	0	0	0	+
14 f55	PTC	0.80	0.1	0	0.1	0	0	+
15 m39	FA	0.95	0	0	0	0.1	0	+
16 m 66	PTC	1.08	4.0	0	0.4	0.2	0	+
17 f 47	PTC	0.68	0.3	0	0.2	0	0	+
18 f48	FTC	0.35	2.8	0	0	0	0	+
19 f53	PTC	0.66	2.4	0	0	0	0	+
20 m 51	PTC	0.88	1.0	0	0	0	0.3	+

Table 3.1 **CELL KINETICS, P53 EXPRESSION AND SYNTHETIC FUNCTION IN A SERIES OF THYROID NEOPLASMS.**

* Case diagnosed at autopsy.

Numbers other than age are % counts per 1000 cells.

ATC = anaplastic thyroid carcinoma

PTC = Papillary thyroid carcinoma

Cases 9-20 represent controls.

FTC = Follicular thyroid carcinoma

MTC = Medullary thyroid carcinoma

FA = Follicular Adenoma

TG = Thyroglobulin

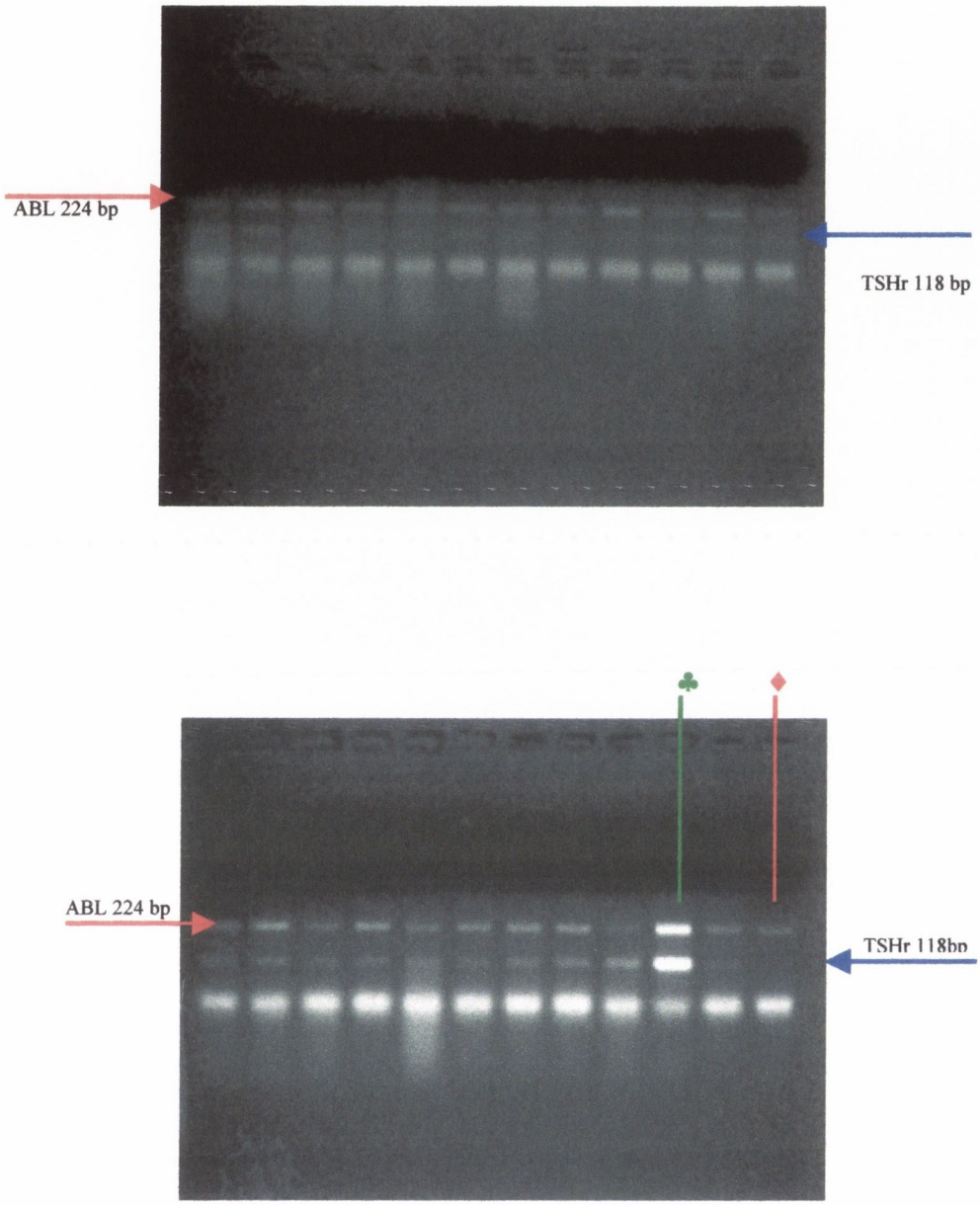
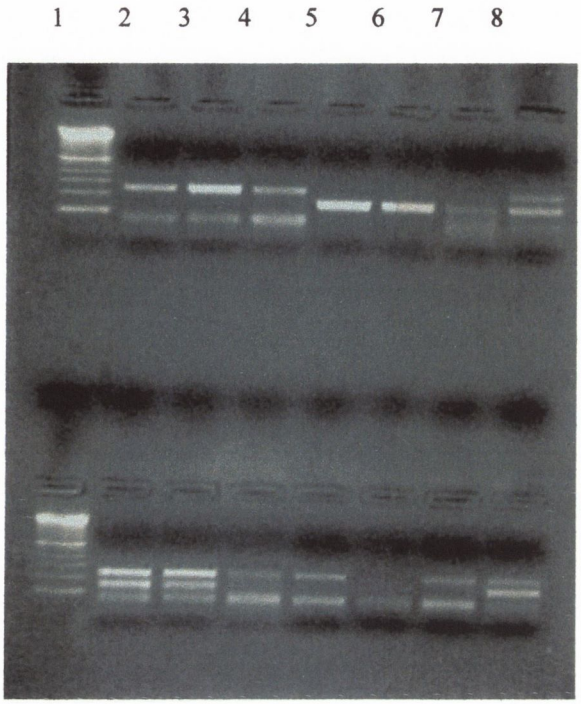


Figure 3.3 Gel electrophoresis demonstrating TSH receptor and ABL amplicons form a group of formalin fixed paraffin embedded thyroid tumours.

2.5% agarose gel electropherogram demonstrating parallel bands for ABL and TSH receptor in a group of thyroid tumours. The intensity of the bands was measured using a Pharmacia LKB-image Master with 'Diversity One' software. Differences between the intensities of the two markers in differeent samples can be appreciated visually (e.g. lanes ♣♦).



Lane Number	Sample	Lane Number	Sample
1	Size marker	9	Size marker
2	ABL sample A (PTC)	10	Multiplex A (PTC)
3	ABL sample B (FTC)	11	Multoplex B (FTC)
4	ABL sample C (ATC)	12	Multiplex C (ATC)
5	TSHr sample A (PTC)	13	Multiplex C(duplicate)
6	TSHr sample B (FTC)	14	Negative control
7	TSHr sample C (ATC)	15	Multiplex C (triplicate)
8	Multiplex (control cell line)	16	Multiplex (control cell line)

Figure 3.4 Gel electrophoresis demonstrating PCR products amplified singly and in multiplex.

3.5 DISCUSSION

Prognostic accuracy in tumour grading and staging is directly proportional to the number of adverse features present. Unfortunately, by the time most readily-appreciable adverse features are present (e.g. nodal metastases or tumour necrosis) prognostication has already become 'deadly accurate'.

Clinical staging and histological grading of tumours are the mainstays of generating objective prognostic data. In recent years however, study of cell kinetics has yielded useful information in prediction of the biological behaviour of neoplasms (Soares et al 1994, Yoshida et al 1992). Grading is the term used for histological assessment of the 'wildness' of a tumour, based upon tissue pattern, cell morphology and proliferation/ necrosis.

Refinements of the criteria used for each of these crude parameters has led to improved prognostication but it remains true that some tumours of identical grade do not behave as expected. Various attempts have been made to estimate growth rate more precisely by features or cell products which are associated with cell growth (Mizukami et al 1993). These include antigens exhibited on the nuclei of proliferating cells such as Ki-67 (Cattoretti et al 1992,1993, Key et al 1993) and PCNA (Shimizu et al 1993, Tateyama et al 1994, Skopelitou et al 1993). Cell membrane products such as EGFr and mutant p53 indicate aberrant and increased sensitivity to growth factors or deregulation of growth suppresser genes respectively. Detection of apoptotic cells, the volume of which might reasonably be negatively correlated with net tumour growth is another technique which may yield useful information with regard to cell growth dynamics.

In this study, three types of proliferation marker were employed to assess the growth fraction in a series of ATC and a control group comprising PTC, FTC, MTC and FA.

Mitotic count has been used extensively to assess prognosis. In this investigation, we demonstrated an increased mitotic rate in ATC (1.1%) compared with the control group (0.13%) in keeping with data from previous studies. However, counting of mitoses in routinely stained histological sections is subjective and may not always be reproducible.

In an attempt to overcome these potential limitations, immunohistochemical staining for Ki-67 and EGFr were also performed. The Ki-67 antibody used reacts with a human nuclear cell proliferation associated antigen which is expressed by all cells except those in G_0. Thus, Ki-67 counts provide an estimate of the total tumour growth fraction. The antibody works successfully on formalin fixed paraffin embedded tissue after antigen retrieval using a citrate buffer and microwave oven (Cattoretti et al 1992, 1993).

Previous studies have focused on PCNA for estimation of proliferative activity. PCNA is expressed in proliferating cells and is present in greatest concentration during S phase (Soares et al 1994, Shimizu et al 1993). Therefore it gives an indication of how rapidly a tumour is growing. In contrast, Ki-67 counts indicate the proportion of tumour cells that are proliferating, or the "growth fraction" (Gerdes 1993,1994). Results of this study have shown an increased level of Ki-67 staining in the ATC group (29.9%) over the controls (1.5%).

Alterations in various growth factors and their receptors have been established as important features of the neoplastic process, among them EGF and its receptor (Duh et al 1985). EGF is known to induce cell proliferation in several tissues.

Recently, EGFr staining has been suggested as an independent predictor of recurrent disease (Akslen 1993). EGFr has not been assessed in a large group of ATC previously. We found a greater degree of staining of EGFr in the ATC cases (median-positivity = 49%) than the control group where staining was negative in all but one case. This was a case of MTC which had widespread metastases from which the patient subsequently died.

The high incidence of EGFr expression in ATC is consonant with the well recognised propensity of these tumours to invade extrathyroidally.

The immunostaining pattern obtained with EGFr is noteworthy, as both the intensity and pattern varied between different tumours. 4/6 ATC expressed diffuse cytoplasmic staining while 1/6 had a discrete membranous pattern. This pattern has been observed previously (Akslen et al 1993) where diffuse cytoplasmic staining has been tentatively linked with extra-thyroidal growth, although this has yet to be clarified.

In breast carcinoma increased expression of EGFr has been related to dedifferentiation of tumour cells, decreased concentration of oestrogen receptors and increased Ki-67 staining. Di Carlo et al (1990) have also demonstrated an inverse correlation between EGFr and TSH response in ATC.

The tumour suppressor gene p53 is alleged to be involved in neoplastic transformation in many human tumours (Lane 1994). In the thyroid gland p53 mutations have been reported to occur with more frequency in ATC than well differentiated carcinomas, and this is confirmed in our results. In normal cells the concentration of wild type p53 is generally below the threshold for detection by immunohistochemical techniques, as it is rapidly eliminated due to its short half life. Mutant p53 protein, produced by malignant cells unlike wild type has a

prolonged half life and therefore may be present at levels detectable by immunohistochemistry.

We demonstrated heterogenous p53 immunoreactivity in all ATC cases (median-cell positivity 44.6%) compared to the control cohort where 1/12 had staining (median-cell positivity 0.05%). However, there was considerable variation among ATC with respect to the percentage of cells which stained positively. This could be due to variables such as fixation, although this is unlikely as the weakly positive p53 cases stained strongly for other antigens. In general, neoplasms with frameshift or missense mutations do not have immunohistochemically detectable p53 due to formation of unstable protein, and this could be another explanation for the variation in positive staining among ATC. The general lack of p53 staining among the controls casts a doubt over its usefulness as a prognostic marker. 4/5 PTC, 3/3 FTC and 2/2 MTC had aggressive metastases and yet had undetectable p53 leading to the conclusion that p53 aberration is more a consequence of dedifferentiation than a causative agent. In addition, we observed an inverse correlation between p53 and thyroglobulin staining, which reinforces the link between p53 and dedifferentiation.

Apoptosis is a key physiological feature which has not previously been examined in thyroid neoplasia. Like mitosis, it is a visible numerator of the cell growth fraction. Correlation of Ki-67 positivity and mitotic counts compared with the apoptotic counts has shown a significantly greater difference in the ATC group than the controls, which ties in with their faster rate of growth. In many of the cases there were apparently more apoptotic bodies than mitoses, which would imply that mitotic count alone is not representative of the proliferative rate in these tumours.

Apoptosis may occur naturally or be potentiated by chemotheraputic agents or irradiation (Kerr et al 1972). It may be useful to identify the subset of ATC which

has a relatively high apoptotic rate as these tumours may be more amenable to non-specific therapy.

TSH is the primary hormone that regulates thyroid cell function and proliferation. There is evidence that TSH stimulates growth and function of differentiated but not anaplastic thyroid carcinomas. Mediation of the effects of TSH is through its interaction with receptors on the cell surface. Loss of TSH receptor expression in thyroid carcinomas would obstruct the efficacy of TSH and lead to uncontrolled growth and loss of differentiated function.

The technique used in this study to detect TSHr expression while not purporting to be quantitative does give a reasonably accurate indication of the trend towards decreased expression of the receptor with loss of differentiation of the tumour. The concept of using PCR based techniques and normalising levels of detection of TSHr against a ubiquitously expressed housekeeping gene will be expanded and developed in the following chapters.

Multiplex PCR was performed for TSHr and Abl. The intensity and area occupied by each band was compared for every case analysed, in an attempt to overcome the variable and uncontrollable effect of RNA degradation prior to extraction.

Levels of TSHr detected were consistently lower in ATC than the control group, thus linking decreased expression of receptor and dedifferentiation. Within the control cohort, there were three cases with lower than average TSHr expression. Two of these were Tall Cell Variant PTC, which is known to be a more aggressive form of PTC. The other was an FTC, which was not remarkable histologically, being negative for p53 and EGFr and with a low Ki-67 count. Interestingly, this patient was readmitted three months after the initial biopsy with bone metastases, indicating a degree of aggression not anticipated with the diagnosis of FTC.

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CHAPTER 4

TSH RECEPTOR STATUS OF THYROID NEOPLASMS – TAQMAN RT-PCR ANALYSIS OF ARCHIVAL MATERIAL.

4.1 Summary

This chapter follows on from the basic gene expression assessment of TSHr performed in Chapter 3. The data obtained in Chapter 3 suggested a correlation between TSHr expression analysis and tumour differentiation. The technology applied and experimental designs utilised in this chapter are more sophisticated and enable more accurate analysis than the preliminary study. The recently described technique of TaqMan PCR is ideal for gene expression studies, and indeed for work with archival material in that the amplicon generated is considerably smaller than conventional solution phase PCR.

Regulation of thyroid follicular cell proliferation and function is mediated by the interaction of TSH with its receptor (TSHr) on the plasma membrane. While it is recognised clinically that responsiveness of thyroid epithelial tumours to TSH varies with the histological type and grade of neoplasm, the level of TSHr expression in these different tumours has not been quantified hitherto. The aim of this study was to provide this information.

Total RNA was extracted from 125 samples of formalin-fixed paraffin embedded thyroid tissue (comprising 48 papillary (PTC), 29 follicular (FTC), 8 anaplastic (ATC) and 5 medullary thyroid carcinomas (MTC) in addition to 35 samples of either follicular adenoma (FA) or normal thyroid tissue. Samples were reverse transcribed and analysed using TaqMan PCR.

TSHr expression was shown to be similar to normal in FA and inversely related to the grade of the majority of thyroid cancers other than MTC. As expected there was negligible expression in these latter tumours.

Reduced expression of TSHr implies decreased responsiveness to TSH manipulation and is therefore a clinically important prognostic indicator in thyroid cancers.

4.2 Introduction

Prognosis in thyroid carcinoma is usually assessed on the basis of criteria which include patient age, and histological type, grade and stage of tumour. It is well recognised, however, that while occasional tumours with adverse histological features, such as necrosis or high mitotic rate, are cured, other seemingly well-differentiated ones may progress to a fatal outcome.

Surgical removal is the mainstay of treatment in thyroid cancer and presumably those high grade tumours which are cured are those of limited spread in which extirpation has been successful. In addition to surgery, in tumours of lower grade, TSH suppression by thyroxine or TSH stimulation in conjunction with I^{131} administration are important medical strategies used to retard the growth of, or ablate, residual or metastatic disease. It is reasonable to assume that only TSHr-rich neoplasms will be susceptible to TSH manipulation and identification of these variants has significant clinical implications.

4.3 Growth factors and thyroid cell growth

An understanding of the interplay of factors involved in human thyroid cell growth and differentiation and of the signalling pathway is an essential prelude to understanding aberration related to thyroid cell transformation. TSH induces human thyroid cell growth at a higher dose than is necessary for induction of differentiated function. Insulin or insulin-like growth factor (IGF1) synergises with TSH to induce thyroid cell growth while maintaining specialised cell function. Insulin and IGF1 are thus either permissive for other factors without being mitogenic themselves or do not inhibit differentiated function. Indeed, TSH has been shown to enhance insulin-

induced auto-phosphorylation of the insulin and IGF1 receptors in rat thyroid cell lines as well as the phosphorylation of the immediate 184kd endogenous substrate (Sun 1991).

Epidermal growth factor (EGF), on the other hand, induces human thyroid cell growth at the expense of loss of differentiated function (Dumont 1991). In some species, TSH does induce EGF receptors on thyroid cells, rendering them more responsive to EGF. The effects of EGF and TSH on differentiation are largely independent of their mitogenic effect.

4.4 Signalling pathways of TSH

TSH activates both the adenylate cyclase and Phospholipase C pathways. The activation of adenylate cyclase and cAMP accumulation stimulates the dissociation of the regulatory and catalytic sub-units of cAMP-dependent protein kinase-A, with subsequent phosphorylation of various cellular proteins. TSH induces or increases tyrosine phosphorylation of at least eleven proteins.

4.5 G Heterodimeric Proteins

The G proteins are a subfamily of the GTP-binding proteins, which includes *ras*. The G proteins are heterodimeric, composed of α,β and γ subunits each encoded by a distinct gene. The α subunit shows structural and functional homology with other members of the GTP binding protein superfamily. It binds guanine nucleotides with high affinity and specificity and has intrinsic GTPase activity. β and γ subunits are non-covalently bound into a dimeric complex, and are required to regulate the function of α subunits, in addition to directing the complex to the plasma membrane.

G proteins couple a diversity of receptors with their effectors by acting as molecular switches activated and deactivated by the GTPase cycle.

4.6 Structure of the thyrotropin receptor gene

Thyrotropin (TSH) is the primary hormone that regulates thyroid cell function and proliferation. Binding of TSH to its receptor activates cyclic AMP (cAMP) and the phosphoinositol cascade through membrane associated G proteins. A family of G protein coupled receptors has been identified whose members are characterised by the common structural feature of seven transmembrane domains known to be involved in signal transduction and in binding small ligands. In contrast to G protein-coupled receptors that bind small ligands, receptors of the group luteinising hormone/chorionic gonadotrophin receptor (LH/CH-r), follicle stimulating hormone receptor (FSHr) and TSHr have a large glycosylated extracellular domain involved in ligand binding. This characteristic makes the pituitary glycoprotein hormone receptors members of a specific subfamily of G protein coupled receptors.

The human TSHr is split into ten exons with sizes of 327,72,75,75,75,78,69,78,189 and >1412 (figure 4.1). Each intron/exon boundary corresponds to a canonical splice consensus sequence. The coding region of the hTSHr gene has been estimated to be in excess of 60kb (Gross 1991).

The human gene of the TSHr displays an original feature in that nine exons encode part of the extracellular N-terminal domain, while the entire transmembrane and intracellular domains are encoded by a single long exon. This gene organisation contrasts with other G protein coupled receptors, which generally are devoid of intronic structures. Gross et al. have consequently suggested that the TSHr has evolved from a proto-receptor gene.

Figure 4.1 Organisation of the human TSHr gene.

Exon/intron organisation of the human TSHr gene. Exons, represented by boxes are numbered from 1-10. Introns are represented by interupted thin lines. The position of the translation initiation (ATG) codon is indicated.

4.7 Aim

Although several studies have shown that TSHr *function* declines with progressive de-differentiation it is not clear whether this is due to reduction in absolute TSHr numbers or defective receptor signalling. This study was undertaken to quantify levels of TSHr in a variety of thyroid neoplasms with a known clinical outcome and to correlate the findings with histological grade.

4.8 Materials and Methods:

125 samples of PTC (48), FTC (29), ATC (8), MTC (5) and FA (13) or normal thyroid (12) accessioned between 1980 and 1998 were analysed. All material had been fixed in 10% buffered formal saline and embedded in paraffin wax. H&E stained sections were reviewed blind by a histopathologist (ECS) and classified according to a recognised system (Rosai 1992).

Observing anti-contaminating procedures, a 20 μm section from each block was placed in a sterile Eppendorf tube. After dewaxing, extraction was performed using a PUREscript RNA Isolation Kit (Gentra Systems Inc. MN, USA) with modification of the protocol which consisted of homogenisation of tissue in lysis buffer using a tube pestle (Scotlabs, UK), incubation of lysate for 3 hours @ 55°C with 10 U Proteinase–K (Finnzymes, Flowgen, UK) and the addition of 20μg glycogen (Boehringer Mannheim, Germany) as a carrier at the RNA precipitation stage.

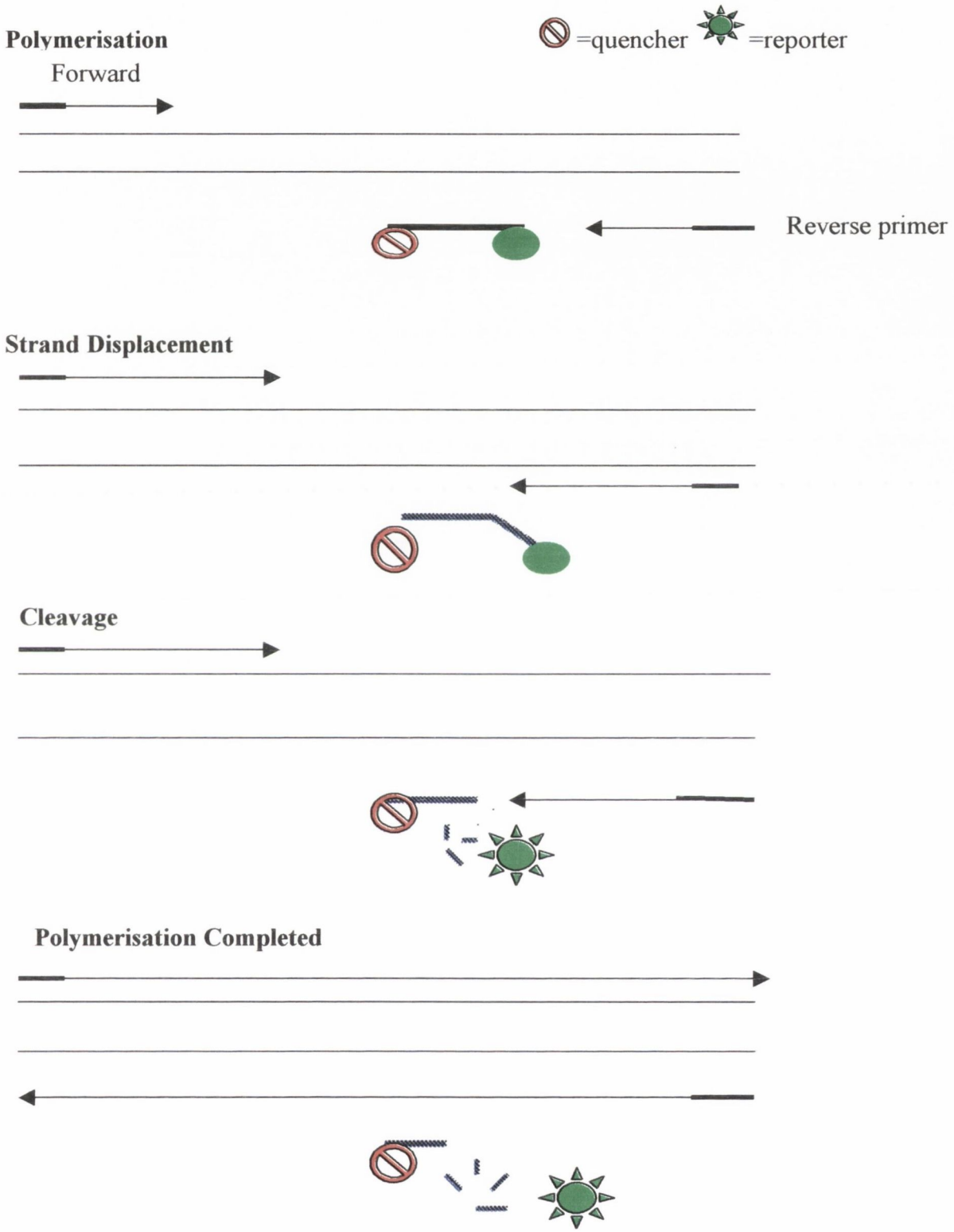
Extracted RNA was reverse transcribed using a GeneAmp RNA PCR kit (Perkin Elmer, CA, USA) with the following reaction conditions: 5mM $MgCl_2$, 1x PCR buffer (Perkin Elmer), 200μM each dNTP, 1U RNase inhibitor, 2.5 U MuLV Reverse Transcriptase, 2.5 μM Random Hexamers. Derived cDNA was used as a template in the TaqMan reaction (figure 4.2).

Primers were designed using Primer Express Software (PE Applied Biosystems) to exon 9 (860F) and exon 10 (933R) with the TaqMan probe traversing the exon junction (881P).
PCR was carried out in a PE 9600 thermal cycler using TaqMan Core Reagents (PE Applied Biosystems) followed by sequence detection using an ABI Prism 7200 Sequence Detector. From cycle 15, fluorescence was measured every 3 cycles to identify the window where amplification was still in the exponential phase.

To compensate for inevitable degradation of target RNA due to tissue processing, duration and conditions of storage, TaqMan PCR was simultaneously performed on aliquots of cDNA from each sample using Glyceraldehyde Phosphate Dehydrogenase (GAPDH) as a housekeeping gene (GAPDH Control Reagents – PE Applied Biosystems). The Rn values obtained for TSHr were divided by the parallel Rn for GAPDH to yield an expression index, TSHr*I*.

A total of 6 negatives ('no template controls') were included in each run along with cDNA from the papillary thyroid carcinoma cell line TPC-1. TPC-1 cells were used as a control source of RNA. The 5 samples of MTC, which would not normally be expected to express TSHr, were also included as a negative control.

Figure 4.2 **5' NUCLEASE ASSAY**



4.9 Results

RNA extraction was performed on a total number of 130 samples in the study. Samples with a negative GAPDH were excluded from further consideration. 125 samples were positive for GAPDH, and TSHr expression was assessed in these cases. The TSHr*I* for the TPC-1 sample was 2.4 +/-0.1 for each run analysed. The results are summarised in the following table.

Table 4.1 TSHr expression in normal and tumour tissue.

Tumour Type	Number of Cases	TSHr*I* Range	Median
ATC	8	1-6	3
PTC (Total)	48	1-26	12
PTC (Well differentiated)	21	11-26	15
PTC Tall Cell Variant	1	14-24	20.5
PTC (Poorly differentiated ± metastases)	26	1-24	4.5
FTC (Total)	29	2-24	10
FTC (Well differentiated)	13	14-24	19
FTC (Well differentiated + metastases)	1	4	4
FTC (Poorly differentiated ± metastases)	15	3-17	3
FA(17)/ Normal(18)	35	11-27	19
MTC	5	3-8	3

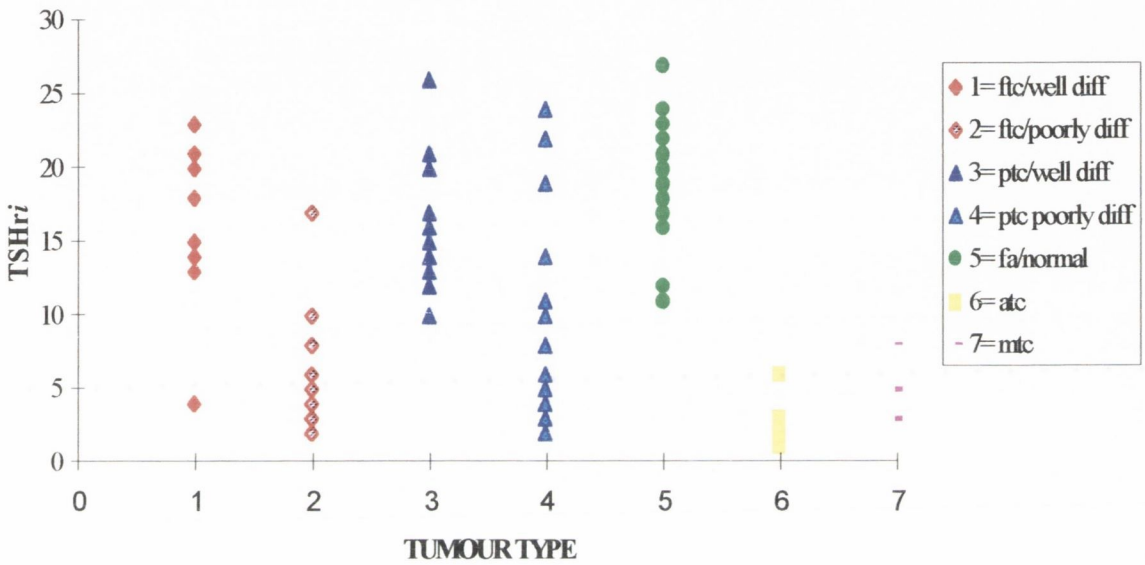
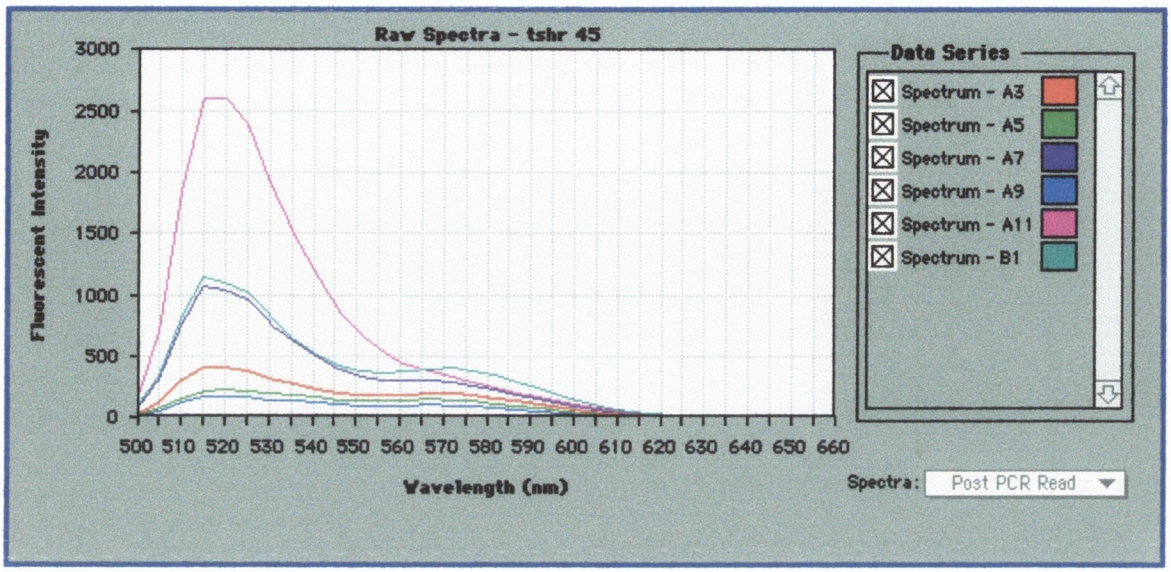


Figure 4.3 TSHr expression in a series of thyroid neoplasms, detected by Taqman RT-PCR.



Colour code for raw spectral printouts:

normal

well-differentiated FTC

well-differentiated PTC

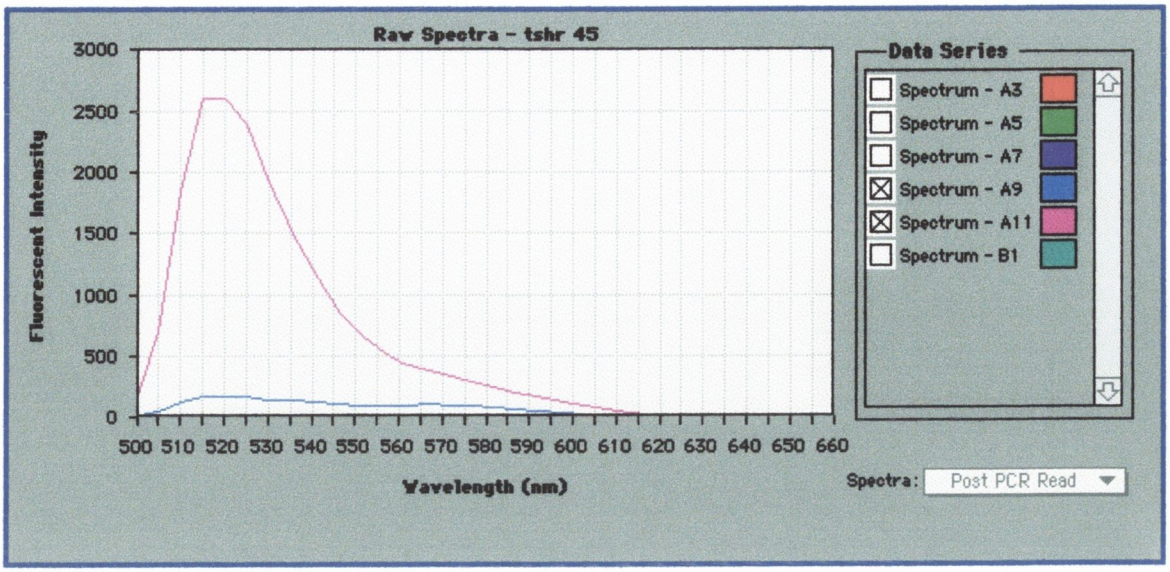
Poorly-differentiated FTC

ATC

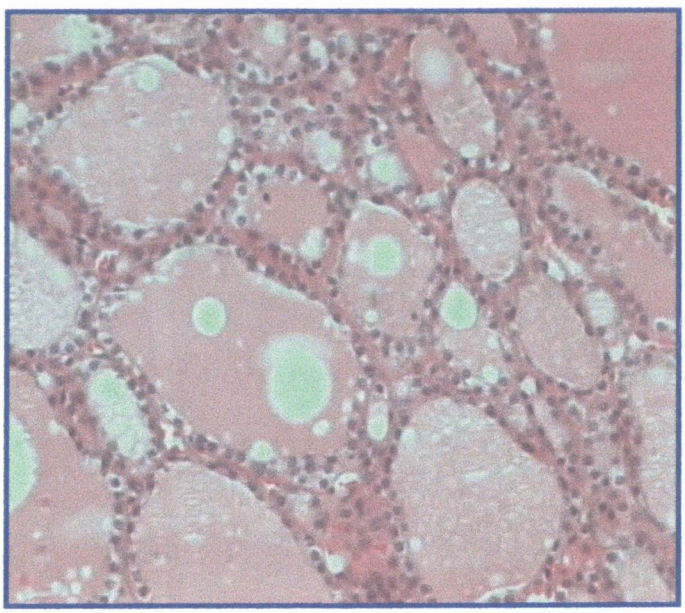
No Template Control

Increased fluorescent emission is detected in more differentiated phenotypes. There is an inverse relationship between TSHr expression and the degree of histological de-differentiation

Figure 4.4 TaqMan Raw Spectra demonstrating TSHr expression in a variety of thyroid neoplasms.

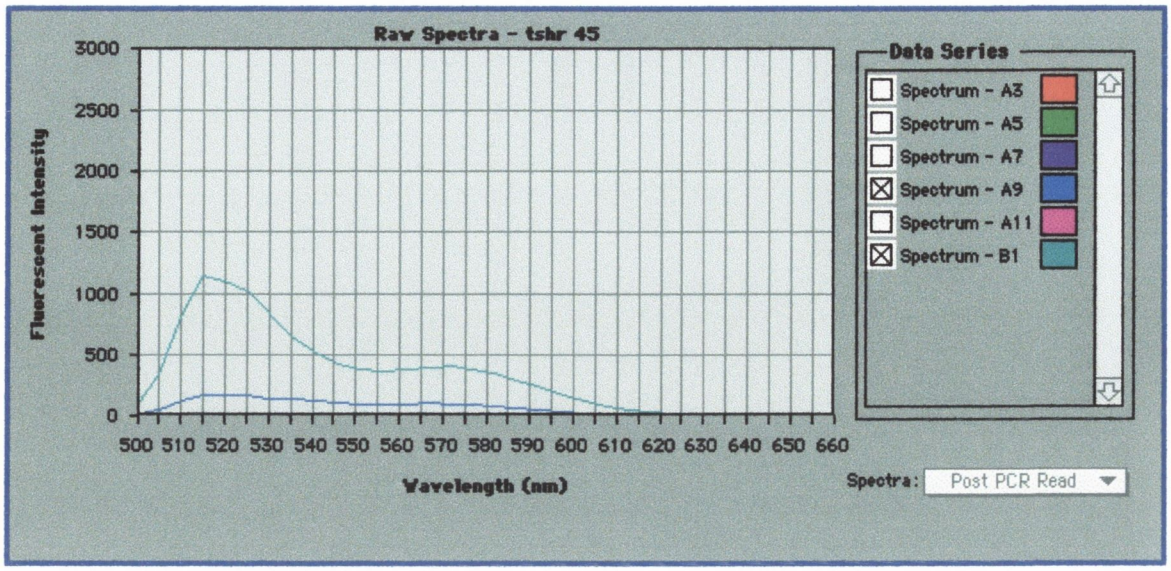


Follicular Adenoma No Template Control

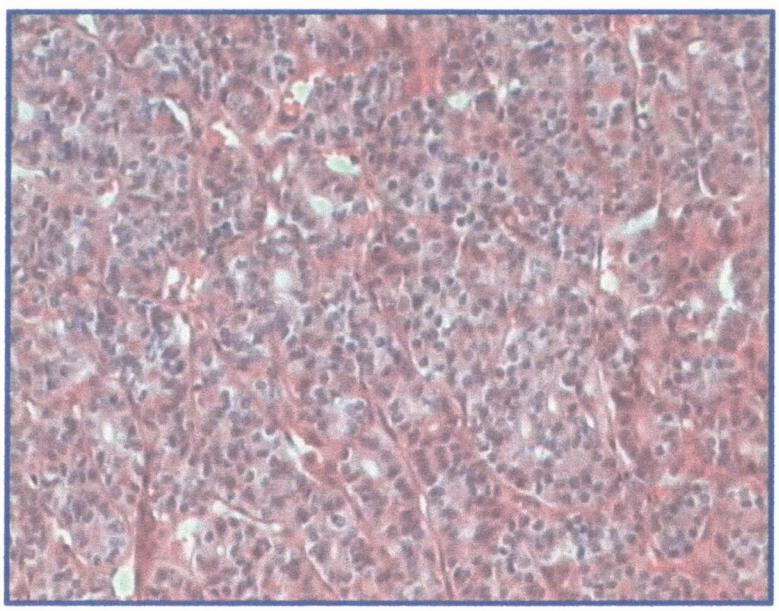


H&E x 25 Follicular Adenoma

Figure 4.5 Raw Spectral printout of TaqMan RT-PCR for TSHr expression in Follicular Adenoma- comparison with corresponding H&E.

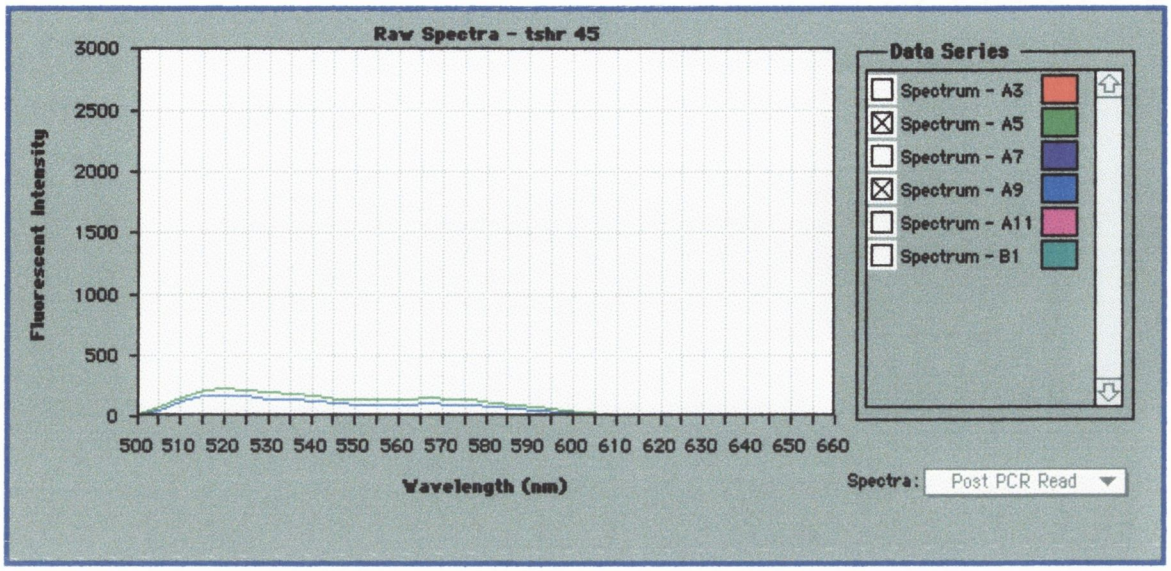


FTC **No Template Control**

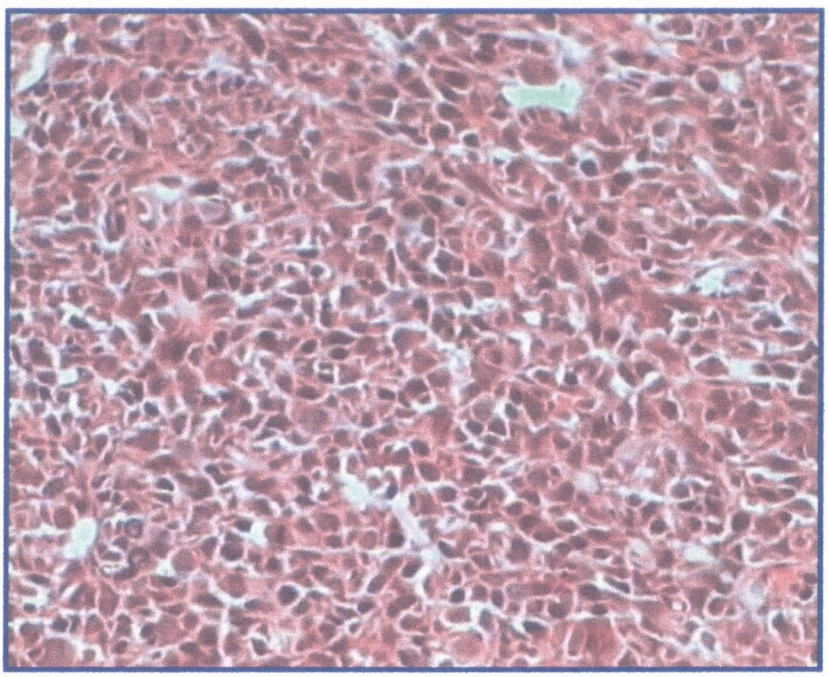


H&E x 25 – Follicular Thyroid Carcinoma

Figure 4.6 Raw Spectral printout of TaqMan RT-PCR for TSHr expression in Follicular Thyroid Carcinoma- comparison with corresponding H&E.



ATC **No Template Control**



H&E x 25 Anaplastic Thyroid Carcinoma

Figure 4.7 Raw Spectral printout of TaqMan RT-PCR for TSHr expression in Anaplastic Thyroid Carcinoma- comparison with corresponding H&E.

4.10 Discussion

The biological behaviour of thyroid neoplasms is, in most instances, directly related to the number of adverse features present (Vassart et al 1993), and in the past clinical staging and histological grading have been the mainstays of prognostication. We were intrigued, however, by a number of cases where the neoplasms failed to conform to their predicted behaviour.

The synthetic function and growth of follicular cells in the normal thyroid are predominantly regulated by TSH through the medium of the TSHr (Nagayama et al 1989, Misrahi et al 1990, Gross et al 1991), a member of the seven transmembrane segment G-protein associated receptors. It has been long recognised that histologically well-differentiated thyroid neoplasms are also responsive to ambient TSH levels, a characteristic which is exploited in the medical management of both benign and malignant lesions. Several studies have shown progressive reduction in TSHr function (Brabant et al. 1991), with increasing de-differentiation of tumours, by means of labelled TSH binding (Ohta et al. 1991) or measurement of TSH associated adenylate cyclase activity (Siperstein A et al. 1988), radioiodine uptake and thyroglobulin synthesis. In addition Northern hybridisation (Shi et al. 1993) and immunohistochemistry (Tanaka et al. 1997) on fresh material demonstrated a strong negative correlation between TSHr expression and tumour stage. However, because the annual incidence of carcinoma of the thyroid is low (Schlumberger et al. 1998) it is difficult to accumulate sufficient fresh material for prospective study by these means.

In contrast, the use of TaqMan (Holland et al. 1991, Livak et al. 1995) RT-PCR allows retrospective analysis of RNA expression for TSHr and has the advantage that, in a reaction where one is dealing with degraded template due to formalin fixation, tissue processing and paraffin embedding, amplicon size is kept to a minimum (typically 50-150bp). Furthermore, to compensate for

possible variations in the level of degradation between samples, parallel amplification of the housekeeping gene GAPDH (assumed to be expressed at a constant level) was performed, to generate a normalised TSHr*I*. This index gives a semi-quantitative figure for the degree of TSHr expression in archival thyroid tissue.

In most instances there was a clear inverse relationship between expression of TSHr and the degree of differentiation of the thyroid neoplasm (figure 4.4). As anticipated, anaplastic thyroid carcinomas had the lowest level of TSHr, with a median expression index of 3, similar to that found in the negative control MTC cases (median= 3) (figure 4.7). By contrast, samples of FA or normal thyroid tissue had consistently high levels of expression (TSHr*I* range = 11 – 27. median=19) (figure 4.5).

Amongst the PTC samples, there was a range of expression, with the well differentiated neoplasms having a TSHr*I* greater than 11. In poorly differentiated PTC the expression index was less than 10 in all instances, bar one. The exception was a tall cell variant PTC, generally accepted as having a poor prognosis (Schlumberger et al.). While no explanation for this is immediately evident, it is interesting to note that the TSHr*I* in this patient declined in a step-wise fashion from 24 to 14 in four successive resection samples accessioned over five years, possibly indicating an increase in tumour aggressiveness with time.

All but one (93%) of the tumours graded as well-differentiated FTC had expression indices ranging from 14 to 24, similar to those found in adenomas and normal thyroid tissue. The outlier was a tumour concealed within a 420g multinodular goitre which presented clinically with a metastatic pathological fracture of the humerus. Despite being microscopically well-differentiated, this neoplasm had a TSHr*I* of only 4, comparable those of the 15 cases in this series histologically graded as poorly differentiated FTC (median = 3, range 3–17) (figure 4.6).

This study indicates that RT-PCR can be performed reproducibly on archival material by means of the TaqMan assay, allowing semi-quantitative assessment of TSHr levels. While the results obtained here demonstrate an inverse relationship between TSHr expression and the degree of histological de-differentiation of most thyroid neoplasms, knowledge of TSHr status in individual cases has important prognostic and therapeutic implications.

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CHAPTER 5

INVOLVEMENT OF *ret*/PTC-1 RE-ARRANGEMENTS IN PAPILLARY CARCINOMA OF THE THYROID.

5.1 Summary

The purpose of this chapter was to design a molecular assay for the detection of activated *ret* proto-oncogene in thyroid carcinoma, and evaluate its incidence. TaqMan RT-PCR was chosen as the assay type due to its ability to give consistent results with formalin fixed archival material. *ret* activation has received much attention in recent years, but its diagnostic implications remain an area of some debate. This is most likely due to variations in technologies applied to its detection (immunohistochemistry → conventional solution phase RT-PCR → TaqMan RT-PCR), and to inter-institutional variation in RNA extraction procedures.

Papillary carcinoma of the thyroid (PTC) has a wide spectrum of biological behaviour. While the majority of cases behave in a clinically indolent fashion, some are highly aggressive and cause death by local invasion or distant metastases.

Among oncogenes studied in thyroid cancers a specific activated form of c-*ret* has been found in a minority of PTC. In these neoplasms, c-*ret* is activated when somatic re-arrangements, occurring within intron 11, juxtapose the intracellular domain of RET to the amino-terminal portion of different donor genes such as H4.

There is contention regarding the clinical implications of c-*ret* activation. Some groups have found age or gender to be important factors when correlating c-*ret* activation, while others have linked activation with metastatic potential or paradoxically less aggressive subsets of thyroid neoplasms. In this study, formalin fixed paraffin embedded tissue from eighty-eight thyroid neoplasms (comprising fifty PTC, eight anaplastic carcinomas (ATC), twenty five follicular thyroid carcinomas (FTC) and five follicular adenomas (FA) for *ret*/PTC-1 and H4 expression using 5' Nuclease Assay (TaqMan RT-PCR). RNA from the TPC-1 cell line was included as a positive control for c-*ret* activation. None of the FTC or FA cohorts displayed activation of *ret*/PTC-1, while H4 was detected in all

these cases. Thirty three percent of all cases (PTC +ATC) were found to express the chimeric RNA characteristic of *ret*/PTC-1. Seven of the eight ATC (87.5%) were positive.

A striking feature of the *ret*/PTC-1 positive PTC cases (twelve of fifty) was the background of chronic inflammation either within the tumour and/or in the surrounding tissue that was observed in the majority (58%). In addition, a failure to express the RNA transcript for the H4 gene was observed in *ret*/PTC-1 positive cases.

The results of this study demonstrate a statistically significant and previously unreported association between PTC which express RET/PTC-1, fail to express H4 and concomitant chronic thyroiditis.

5.2 Introduction

The prognosis for patients with PTC is generally dependent upon age and tumour stage at time of diagnosis (Schlumberger 1998). Nonetheless, the biological aggressiveness of individual tumours cannot always be predicted from the initial clinical features, making it difficult to consistently identify patients who will die from their disease.

A variety of prognostic markers such as proliferative (Sugitani 1998), mitotic and apoptotic indices, EGF receptor (Westermark 1996) and TSH receptor (Sheils 1999) expression have been studied with a view to more accurate prediction of the outcome of a given thyroid carcinoma.

c-*ret* is one of a myriad of proto-oncogenes which has been examined to assess the prognostic implications of its activation in thyroid disease. *ret*/PTC is an activated form of c-*ret* which has been found exclusively in papillary thyroid carcinoma (Grieco 1990, Santoro 1993).

5.3 Consequences of oncogene expression.

The epidemiology of cancer in populations strongly suggests that neoplastic transformation of cells *in vivo* requires the accumulation of several genetic lesions. In humans, the multi-step nature of neoplasia may be readily distinguished in some forms of disease. In cervical cancer the initiating event is probably infection with human papillomavirus. Subsequently, cervical dysplasia (cervical intraepithelial neoplasia, CIN) may arise. CIN types 1,2 and 3 represent progressively more severe forms in the development of malignant cervical carcinoma. The fact that smoking significantly increases the probability of cervical tumour progression indicates the importance of environmental factors.

5.4 The *ret* proto-oncogene and cancer

RET (**RE**-arranged during **T**ransfection), as its acronym suggests, was cloned as a chimeric oncogene during a classical NIH3T3 transformation assay (Takahashi 1985). The *ret* proto-oncogene codes for a receptor-like tyrosine kinase (TRK) whose extracellular domain contains a conserved cysteine rich region close to the cellular membrane and a more distal cadherin -like domain (Schneider 1992).
The *ret* proto-oncogene, a receptor kinase, has been evaluated as a candidate gene for multiple endocrine neoplasia type 2A and type 2B (MEN 2A and MEN 2B), for familial medullary thyroid carcinoma (FMTC), and for sporadic cases of medullary thyroid carcinoma (MTC) and phaeochromocytomas.
ret has been mapped to the centromeric region of chromosome 10q11.2, and its physiological expression is restricted to cells of neural crest origin.

However, the *ret* proto-oncogene is not only involved in the development of neuro-endocrine tumours. Several investigators have linked papillary thyroid carcinomas with chromosomal re-arrangements of the *ret* gene. To date three varieties of *ret* re-arrangement have been described, with *ret*/PTC-1 being the most commonly detected (≈70%). These re-arrangements result in an mRNA chimera in which the tyrosine kinase domain of RET is fused with a donor sequence (Williams 1995). The membrane anchoring and ligand binding domains of RET are lost in this process, while the 5' sequences that donate their promoters become juxtaposed to RET. These donor proteins are ubiquitously expressed in their normal form.

5.5 Mutations in *ret.*

Despite its unknown physiological function in humans, *ret* is involved in determining at least five different disease phenotypes. Germline point mutations in one of five cysteines of the extracellular domain (exons 10 and 11) have been detected in 97% of patients with MEN 2A and 86% of patients with FMTC

(Donnis-Keller 1993). Moreover, a single mutation in codon 918 substituting a threonine for a highly conserved methionine in the catalytic core region of the tyrosine kinase domain is present in almost all patients with MEN2B (Carlson 1994). These mutations which have been shown to activate the *ret* proto-oncogene as a dominant transforming gene (Asai 1995) also occur somatically in a number of cases of sporadic medullary thyroid carcinoma and phaeochromocytoma (Eng 1994).

In addition, heterozygous deletions of ret or frameshift, nonsense or missense mutations scattered throughout the entire *ret* coding sequence are associated with the dominant form of Hirschsprung disease or congenital colonic aganglionosis (Yin 1996). A loss of function effect has been demonstrated in three missense mutations of Hirschsprung disease to date (Pasini 1996).

Finally, the *ret* proto-oncogene is activated in a number of cases of papillary thyroid carcinoma involving somatic re-arrangements within intron 11.
Molecular characterisation of *ret*/PTC-1 has shown it to be generated by fusion of the tyrosine kinase encoding region of c-*ret* to the 5' terminal sequences of a gene termed H4 (Grieco 1994). It is believed that RET/PTC fusion proteins may cause cell transformation by reactivation of the foetal c-RET kinase domain which is found in all chimeric RET proteins (Pasini 1995, Tong 1995).

5.6 Proto-*ret* and proto-*trk*

The proto-oncogenes *ret* and *trk* encode tyrosine kinase membrane receptors whose physiological function appears related to the differentiation of particular components of the peripheral nervous system. Their constitutive ectopic expression has been detected in approximately 50% of human papillary thyroid carcinomas activated by chromosomal re-arrangements.
These two relevant proto-oncogenes share a significant number of features:

(a) their physiological expression is restricted to cells of neural crest origin (Szentirmay 1990).

(b) chromosomal re-arrangements form hybrid molecules which display constitutively activated RET and TRK-derived tyrosine kinase domains.

(c) they comprise a super-family of oncogenes whose expression is restricted to papillary thyroid carcinoma.

TRK and RET oncoproteins having lost their original signal peptide and being re-arranged with non-membrane bound proteins are located in the cytoplasm where their transforming effect is activated.

5.7 Comments on *ret*/PTC-1 positive PTC.

In this study, chronic lymphocytic inflammation was present in 58% of those thyroids harbouring PTC positive for *ret*/PTC-1. Failure to express H4 was also noted in this cohort. It has been suggested that some thyroid cancers may induce an immunological response similar to that seen in thyroiditis (Sibbit 1991) because the antigenic profile of the cells has been altered in the process of neoplastic transformation. Alternatively, loss of expression of another involved protein may induce an 'altered self' state which can induce an immune response. In this regard it is of interest that *ret*/PTC-1 and/or *ret*/PTC-3 expression has been found recently in 95% of Hashimoto thyroiditis cases in which co-existing PTC had been excluded (Wirtschafter 1997).

5.8 Materials and Methods

Eighty-eight thyroid neoplasms comprising eight anaplastic (ATC), fifty papillary (PTC), twenty five follicular thyroid carcinomas (FTC) and five follicular adenomas (FA) accessioned between 1986 and 1998 were included in the study. All tissues were fixed in 10% formal saline and embedded in paraffin wax. Diagnosis was confirmed on H&E stained sections in all cases.

Attention was given to standard anti-contaminating procedures. A single 20μm section was cut from each block and placed in a sterile Eppendorf tube. Sections were dewaxed in xylene, and RNA extracted using a PUREscript RNA Isolation Kit (Gentra Systems Inc. MN, USA), using previously described modifications. Reverse transcription of extracted RNA was performed using a GeneAmp RNA PCR kit (Perkin Elmer, CA, USA) following the supplied recommended reaction conditions. The derived cDNA was used as a template in the TaqMan reaction. Primers designed using Primer Express Software (PE Applied Biosystems) were as follows:

ret/PTC-1 F	5'	CGC GAC CTG CGC AAA	3'
ret/PTC-1 R	5'	CAA GTT CTT CCG AGG GAA TTC C	3'
ret/PTC-1 Probe	5'	CCA GCG TTA CCA TCG ACC ATC CAA AGT	3'
H4 – F	5'	AGG AGG AGA ACC GCG ACC	3'
H4 – R	5'	CTT CCT GCT CAG CCC TGG	3'
H4 – Probe	5'	CGC AAA GCC AGC GTT ACC ATC CA	3'

Primers for *ret*/PTC-1 detection were designed to flank the junction between the tyrosine kinase encoding region of c-*ret* and the 5' terminal sequence of H4, while those for H4 flanked the junction between exons 1 and 2 of H4. Probes were designed to span the exon-exon boundary for H4, and the *c-ret*-H4 fusion point for *ret*/PTC-1.

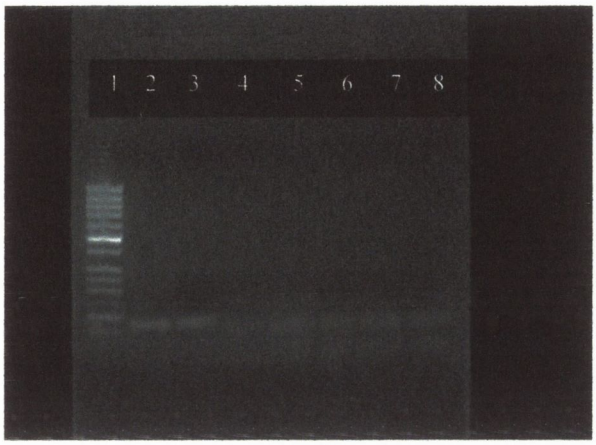
cDNA from the TPC-1 cell line (Jossart 1995) was used as a positive control for *ret*/PTC-1 re-arrangement. Primers and TaqMan probe for the housekeeping gene Glyceraldehyde Phosphate Dehydrogenase (GAPDH) were used to confirm the integrity of extracted RNA. PCR was carried out using a PE 9600 thermal cycler using TaqMan Core Reagents (PE Applied Biosystems) followed by sequence detection using an ABI Prism 7200 Sequence Detector.

5.9 Results

ret/PTC-1 chimeric transcripts were detected in nineteen of eighty-eight tumours, in addition to the control cell line (TPC-1). None of the FTC or FA samples had detectable *ret*/PTC-1 activation, while H4 transcripts were detected in all cases in these categories. Of the eight ATC cases, seven had detectable *ret*/PTC-1, while twelve of fifty PTC were positive. Among the positive PTC samples seven occurred in tumours with a background of chronic thyroiditis, while only two of the *ret*/PTC-1 negative tumours displayed this feature ($p=0.0002$ Fisher's Exact Test) (table 5.1).

H4 expression was detected in all the *ret*/PTC-1 negative cases. Of the seven *ret*/PTC-1 positive ATC cases, six had no detectable expression of H4. Eleven of the twelve *ret*/PTC-1 positive PTC cases failed to express H4.

Successful amplification of the extracted RNA was achieved in all cases, demonstrated by positive signals for GAPDH. To demonstrate the increased sensitivity of the TaqMan assay over conventional solution phase RT-PCR, TaqMan products were electrophoresed on 1.5% agarose gels, and the presence or absence of visible bands were compared with TaqMan Rn (figure 5.1).



Lane 1= 50 bp size marker

Lane 2,3,5,6,7,8 = samples positive for ret/PTC-1 using Taqman RT-PCR.

Lane 2= TPC-1 cell line

Lane 3= PTC

Lane 5= ATC

Lane6= PTC

Lane 7= PTC

Lane 8= ATC

(see raw spectra below for emission patterns relating to lanes on gel).

Lane 4 = negative control

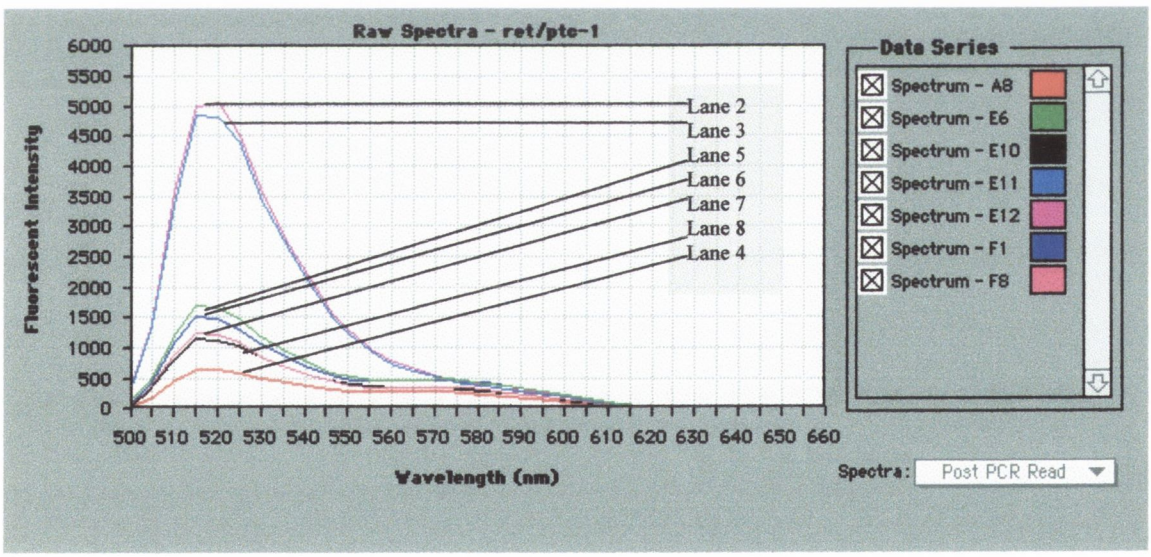


Figure 5.1 Comparison between agarose gel electrophoresis of solution phase RT-PCR amplicons and TaqMan RT-PCR.

	ret/PTC-1 positive	ret/PTC-1 negative	Total number of cases
Presence of chronic lymphocytic inflammation	7 (14%)	2 (4%)	9 (18%)
Absence of inflammation	5 (10%)	36 (72%)	41 (82%)
Total number of cases	12 (24%)	38 (72%)	50 (100%)

Fisher's Exact test

The two-sided P value is 0.0002, considered extremely significant.

There is significant association between rows and columns.

Table 5.1 Comparison between ret/PTC-1 positivity and presence of inflammation.

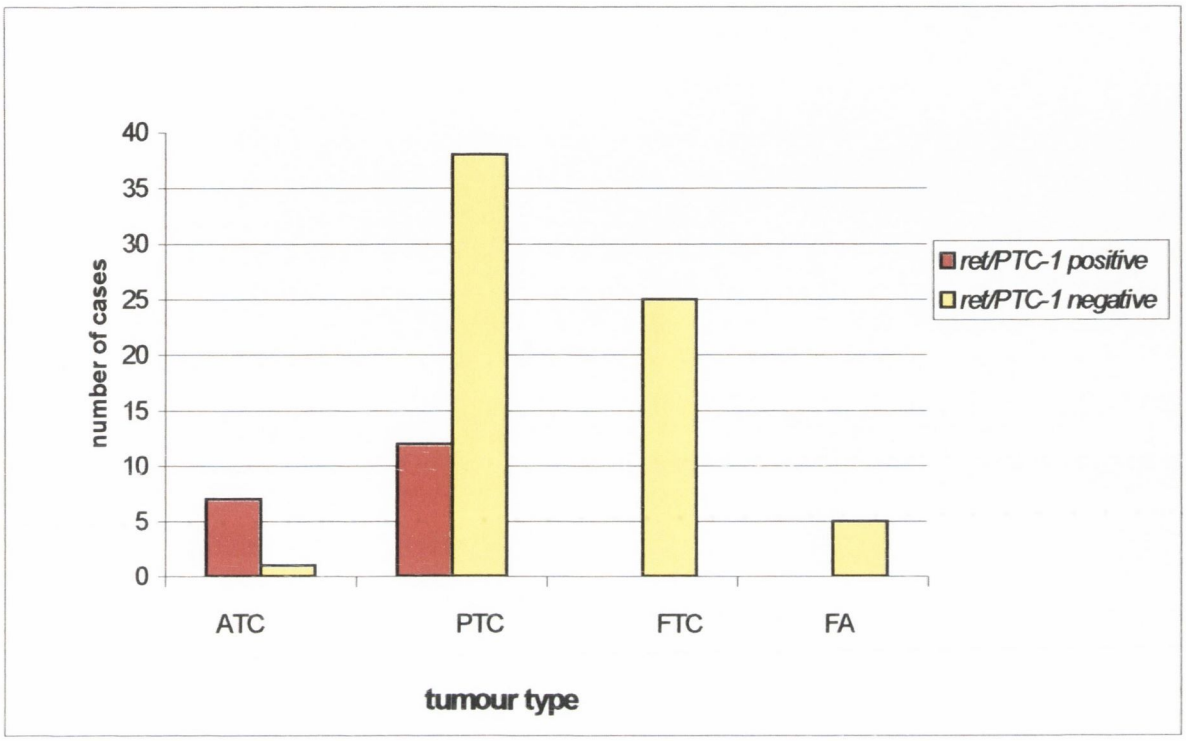


Figure 5.2 ***ret*/PTC-1 Positivity in Thyroid Carcinoma**

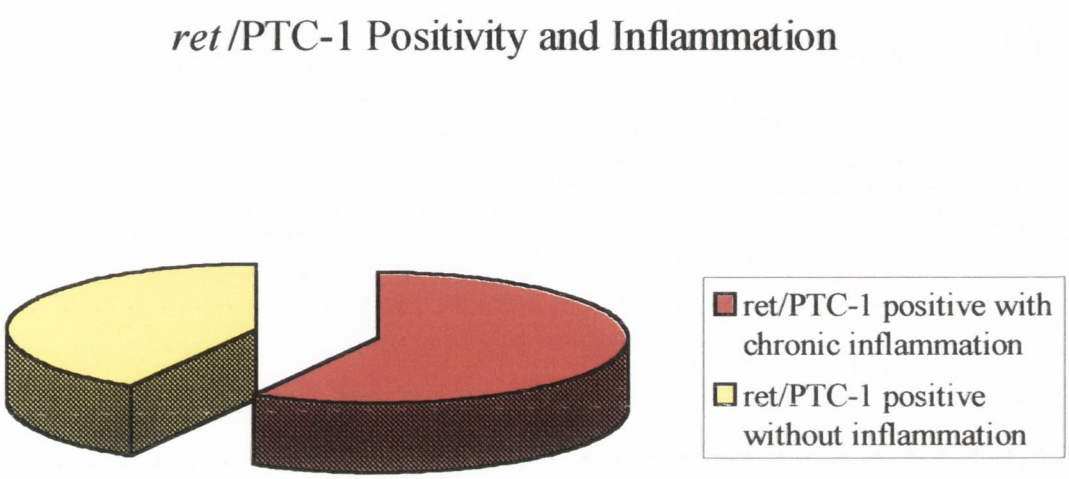


Figure 5.3 Chart depicting ret/PTC-1 positive cases with associated inflammation

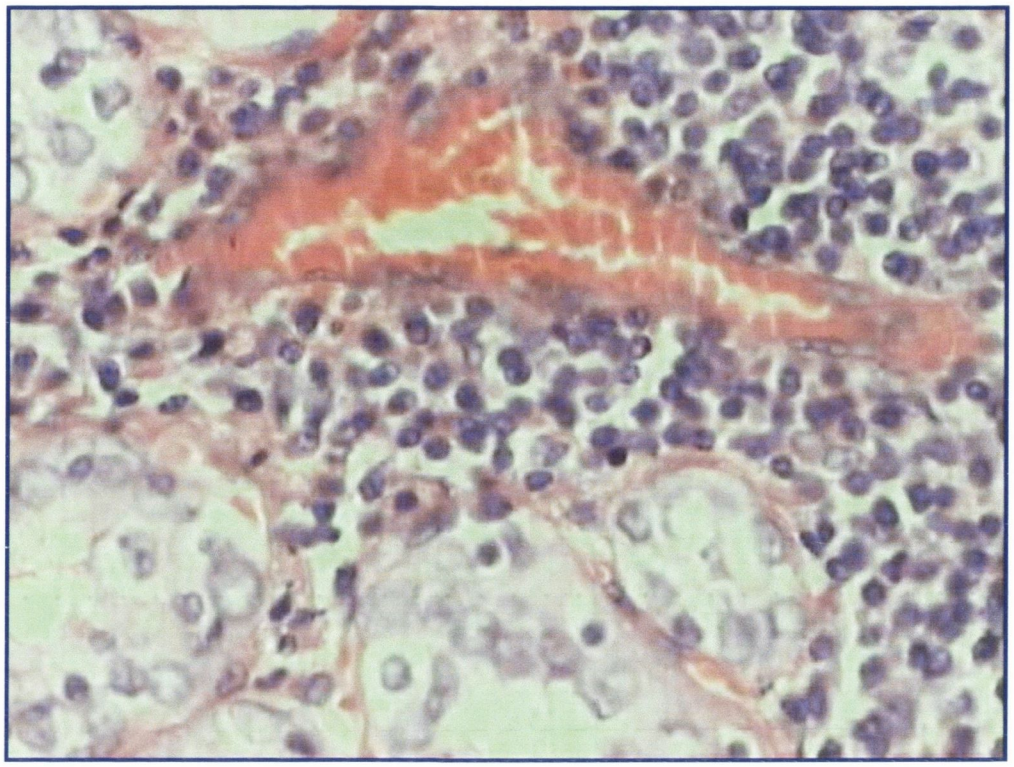


Figure 5.4 H&E stained section of *ret*/PTC-1 positive PTC with associated thyroiditis.

5.10 Discussion

PTC is the commonest variety of thyroid carcinoma representing 70% or more of total cases. While the majority of PTC are slow growing and clinically indolent, some are highly aggressive and rapidly produce local invasion or distant metastases.

Recently, several chromosomal translocations and inversions involving the c-*ret* proto-oncogene have been documented in PTC. Abnormalities of c-*ret* in thyroid were first described in analyses of thyroid carcinoma DNA using the NIH-3T3 transformation assay. Activation is provoked by genetic re-arrangement where the tyrosine kinase domain of the oncogene is juxtaposed on a second gene. This fusion culminates in the expression of a chimeric protein, which displays a constitutive tyrosine kinase activity, with the loss of the ligand binding and transmembrane domains of RET.

To date three varieties of c-*ret* activation have been described, with *ret*/PTC-1 occurring most frequently. This variant comprises the tyrosine kinase domain of c-RET and a partial sequence of a gene termed H4. It has been suggested that the protein encoded by H4 may function as a cytoskeletal protein (Grieco 1994). Such re-arrangements of the *ret* proto-oncogene resulting in activation are highly specific for PTC *in vivo*. However, the clinical implications of *ret* activation remain controversial.

In this study, we examined a series of 88 thyroid tumours comprising eight ATC, fifty PTC, twenty five FTC and five FA using TaqMan RT-PCR for the presence of *ret*/PTC-1 and H4 transcripts. The TaqMan technique has been described in detail elsewhere (Sheils 1999). PCR primers for the *ret*/PTC-1 assay were designed such that they bordered the junction between the tyrosine kinase encoding region of c-*ret* and the 5' terminal sequence of H4. The TaqMan probe was designed to traverse the junction point. The incorporation of the probe provides an extra dimension of specificity to the reaction, while also eliminating

the detection of non-specific amplification due to mis-priming or primer-dimer artifacts. The TaqMan assay is extremely sensitive and has been shown capable of discriminating two transcripts among 10^5 contaminating sequences.

The demonstration of *ret*-PTC-1 expression in the anaplastic subtype of thyroid carcinoma is at variance with the results of a study by Soares et al (1998), where they concluded that *ret*/PTC-1 activation detected by Southern analysis was associated with a lack of aggressiveness in PTC. In addition the findings of Tallini et al (1998) using solution phase RT-PCR supported the association between *ret*/PTC positive PTCs and failure to progress to more aggressive phenotypes.

Our detection of *ret*/PTC-1 activation in the aggressive ATC group may be accounted for by the relative insensitivity of conventional solution phase RT-PCR when compared with the highly sensitive and extremely specific TaqMan technique. Indeed, when conventional solution phase RT-PCR was applied to the samples in this study cohort, using the same primers but without integrating the fluorescent labelled probe, we noted a failure to detect signals for GAPDH and ret/PTC-1. While the histogenesis of ATC is not necessarily PTC, 20% have a history of differentiated carcinoma and a further 20-30% have concurrent differentiated tumour frequently papillary in nature (The Endocrine System-Robbins Pathologic Basis of Disease). It is well recognised that the cumulative effects of a series of genetic insults are required to achieve de-differentiation.

Consequently, among the ATC cohort, activation of *ret*/PTC-1 may represent one of many events that have occurred at a genetic level, unlike PTC where it appears to be the significant mutation. Using quadrant geographical sampling of selected cases, we have detected *ret*/PTC-1 transcripts in both papillary and anaplastic phenotypes of the same tumour. To elucidate this further, it would be interesting to quantitate and compare the level of expression of the chimeric transcript in ATC versus PTC.

Other groups have found *ret*/PTC-1 activation in association with aggressive varians of thyroid cancer (Jhiang S 1994, Sugg 1996, Klugbauer 1995). Our results are in accord, for example, with the findings of Bongarzone et al (1998) who demonstrated an increase in expression of RET and /or NTRK transcripts in radiation induced PTC. They concluded these features were associated with increased aggressiveness in tumours with respect to stage, and specifically linked *ret*/PTC-1 activation with locally advanced stage.

Perhaps the most striking observation in this study was the high proportion of *ret*/PTC-1 positive tumours associated with a background of chronic thyroiditis (58%), compared with 4% of *ret*/PTC-1 negative cases (p=0.0002-(table 5.1)). H4 (D10S170) is a gene whose predicted protein does not have a transmembrane domain and which shows extensive alpha helical conformation. These regions have 30% homology with those alpha-helical regions of proteins such as tropomyosin, vimentin and keratin. Consequently, it has been suggested that its function may be cytoskeletal protein synthesis (Grieco 1994). Among those cases, which expressed the chimeric transcript for *ret*/PTC-1, 90% failed to express H4 as detected by TaqMan RT-PCR.

It is interesting to speculate that RET/PTC-1 expression might be responsible not only for the structural and nuclear peculiarities of PTC (e.g. papillary configuration and optically clear nuclei) but also the induction of an immunological reaction to thyroid epithelium. In relation to the former hypothesis Fischer et al (1998) have demonstrated alterations in the nuclear envelope and chromatin structure in primary culture of thyroid cells infected with a retroviral vector expressing activated RET/PTC-1, although the precise mechanism whereby the nuclear changes take place remains unclear.

In addition, *ret*/PTC-1 activation may influence the growth pattern in PTC, firstly because failure to express the cytoskeletal protein H4 alters the structure of the cell or, secondly, because RET/PTC-1 is constitutively phosphorylated. Yap et al (1997) have shown that increased tyrosine phosphorylation alters thyroid epithelial

organisation in culture by altering the assembly of actin-associated adhesive junctions. Under the influence of tyrosine phosphorylation, thyroid cells lose their capacity to form follicles and spread and migrate into confluent monolayers. It is possible therefore, that similar cytomorphological alterations occur in-vivo in association with *ret*/PTC-1 activation and may contribute at least in part to the adoption of a papillary growth pattern with 'orphan Annie' nuclei.

Although it is well recognised that non-specific lymphocytic thyroiditis may occur adjacent to thyroid tumours (Oertel 1991) the highly statistically significant association between PTC with RET/PTC-1 expression (and concomitant H4 negativity) and chronic thyroiditis has not been documented previously. The possibility that antigenic alterations engendered by activation of c-*ret* (such as loss of ligand binding and transmembrane domains or adhesive junctions) are responsible for inducing an immune response remains speculative. Additional support for the concept of *ret*/PTC-1 activation playing a central role in this phenomenon is provided by the observations of Wirtschafter et al (1997), who found mRNA expression for *ret*/PTC-1 and /or *ret*/PTC-3 in 100% of patients with Hashimoto thyroiditis. It is possible that the intricate association of tumour cells and lymphoid stroma may provide a growth advantage to this subgroup of tumours. In this regard, further work remains to be done to establish whether the lymphoid infiltrate is present in response to cellular alteration due to transformation, or acts as a facilitator to tumour growth.

In this study, chronic thyroiditis was present in 58% of those PTC positive for *ret*/PTC-1 activation, and negative for H4 expression. Distortion of the antigenic profile may result from neoplastic transformation and this may induce an immunological response similar to that seen in thyroiditis. Further investigation at the molecular level may elucidate not only the genetic basis for neoplastic transformation in PTC but also a mechanism for the induction of non neoplastic thyroiditis.

5.11 References

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CHAPTER 6

RET/PTC-1 ACTIVATION IN HASHIMOTO THYROIDITIS.

6.1 Summary

Activation of *ret*/PTC-1 has been documented in a minority of papillary thyroid carcinomas (PTC). The *ret* proto-oncogene is located on chromosome 10 and when c-*ret* is activated, somatic rearrangements occurring within intron 11 juxtapose the intracellular domain of RET to the amino-terminal portion of different donor genes such as H4. This fusion culminates in the expression of a chimeric protein, which displays a constitutive tyrosine kinase activity, with loss of the ligand binding and transmembrane domains of RET. Associated with the acquisition of RET/PTC-1 expression is a concomitant loss of expression of H4. It has been suggested that the product encoded by H4 may function as a cytoskeletal protein.

In the last chapter the presence of *ret*/PTC-1 in association with a background of florid lymphocytic thyroiditis (LT) in 58% of cases of PTC studied was described. Consequently, we were prompted to examine the incidence of RET/PTC-1 expression in 27 examples of various forms of lymphoid infiltration of the thyroid using TaqMan RT-PCR. Overall, twenty-one cases (78%) were found to express the chimeric transcript of *ret*/PTC-1. Eighteen cases of Hashimoto thyroiditis were positive (95%), and of these three had concomitant PTC while the remainder had no histological evidence of associated malignancy. Three cases of lymphocytic thyroiditis demonstrated activated *ret*/PTC-1 (43%), two having associated PTC.

These data indicate that either *ret*/PTC-1 is an indicator of follicular thyroid cell activation, or, that *ret*/PTC-1 activation is implicated as an early event in malignant transformation. If the latter is the case, it suggests that, in a defined sub-set of the population, *ret*/PTC-1 activation elicits an auto-immune response, which, while possibly curtailing the development of PTC in the majority of cases results in destruction of the thyroid parenchyma.

In addition, a new technique: in-situ TaqMan RT-PCR, is described in this chapter. This technique has been applied to the TPC-1 cell line with a view to visualising the chimeric ret/PTC-1 mRNA transcript within the cell. The method was successful in demonstrating the chimeric transcript of ret/PTC-1. The transcript was contained within the cytoplasm. In areas where cells retained follicular structure, the transcript was localised to the luminal surface, where it formed a discrete linear border.

The pattern of localisation detected infers an association between the expression of *ret*/PTC-1 with morphological features in PTC.

6.2 Introduction

Hashimoto thyroiditis is by far the most common form of thyroiditis, with a female incidence some 20 times that of males (The thyroid gland in: Oxford Textbook of pathology 1992). The majority of patients are hypothyroid on presentation with elevated serum TSH levels. The histological changes of diffuse lymphoplasmacytic infiltration of the thyroid with germinal centre formation, Hürthle cell change and variable fibrosis are well recognised. The trigger, which induces the observed auto-immune response in these patients, is unknown.

Recently, several chromosomal translocations and inversions involving the c-*ret* proto-oncogene have been documented in papillary thyroid carcinoma (PTC) (Grieco 1990). Activation is engendered by genetic rearrangement where the tyrosine kinase domain of the oncogene is juxtaposed to a second gene.
There have been three varieties of c-*ret* activation described thus far, with *ret*/PTC-1 occurring most frequently. This variant comprises the tyrosine kinase domain of c-RET and a partial sequence of a gene termed H4 (Grieco 1994).

The possibility that antigenic alterations engendered by activation of c-*ret* (such as loss of ligand binding and transmembrane domains or adhesive junctions) are responsible for inducing an immune response has been postulated (Grieco1994). In this study, using TaqMan RT-PCR 95% of Hashimoto thyroiditis tissues showed evidence of *ret*/PTC-1 activation. The observations of Wirtschafter et al. (1997) who found mRNA expression for *ret*/PTC-1 and /or *ret*/PTC-3 in 100% of patients with Hashimoto thyroiditis using RT-PCR support the concept of *ret*/PTC-1 activation playing a central role in this phenomenon.

6.3 Materials and Methods

6.3.1 TaqMan RT-PCR

Twenty-seven thyroiditis cases accessioned between 1992 and 1998 were included in the study. All tissues were fixed in 10% formal saline and embedded in paraffin wax. Diagnosis was confirmed on H&E stained sections in all cases. In this instance we studied nineteen cases of Hashimoto thyroiditis (HT), three of whom had been found to have occult PTC, five cases of non-specific lymphocytic thyroiditis (LT), two cases of LT associated with PTC and one case of Graves disease with heavy lymphoid infiltration of the gland. Cases were analysed for the presence of ret/PTC-1 activation using RT-TaqMan PCR.

As previously described (Sheils 1999), attention was given to standard anti-contaminating procedures. A single 20μm section was cut from each block and placed in a sterile Eppendorf tube. Sections were dewaxed in xylene, and RNA extracted using a PUREscript RNA Isolation Kit (Gentra Systems Inc. MN, USA), using previously described modifications.

Reverse transcription of extracted RNA was performed using a GeneAmp RNA PCR kit (Perkin Elmer, CA, USA) following the supplied recommended reaction conditions. The derived cDNA was used as a template in the TaqMan reaction. Primers designed using Primer Express Software (PE -Biosystems) were as follows:

ret/PTC-1 F	5'	CGC GAC CTG CGC AAA 3'
ret/PTC-1 R	5'	CAA GTT CTT CCG AGG GAA TTC C 3'
ret/PTC-1 Probe	5'	CCA GCG TTA CCA TCG ACC ATC CAA AGT 3'

Primers for *ret*/PTC-1 detection were designed to flank the junction between the tyrosine kinase encoding region of c-*ret* and the 5' terminal sequence of H4. The probe was designed to span the *c-ret*-H4 fusion point for *ret*/PTC-1.

cDNA from the TPC-1 cell line was used as a positive control for *ret*/PTC-1 rearrangement. Primers and TaqMan probe for the housekeeping gene Glyceraldehyde Phosphate Dehydrogenase (GAPDH) were used to confirm the integrity of extracted RNA. PCR was carried out using a PE 9600 thermal cycler using TaqMan Core Reagents (PE Applied Biosystems) followed by sequence detection using an ABI Prism 7200 Sequence Detector.

6.3.2 In-situ RT-TaqMan

This newly described technique was applied to cytospin preparations of the TPC-1 cell line. Preparations were made onto APES coated slides specifically designed for PCR-ISH (PE Biosystems). The cell preparations were air-dried, and then fixed in Permeafix (Ortho Diagnostics). The preparations were brought through increasing concentrations of ethanol and stored at –70°C in 100% ethanol until staining was performed.

The optimised reaction conditions are described in Chapter 2. The TaqMan reaction was performed for 25 cycles. Negative controls included were as follows:

a) All reaction components @ 0 cycles.
b) No rTth enzyme – 25 cycles
c) No primers – 25 cycles
d) No probe – 25 cycles.

Cells were counterstained and mounted with Vectashield mounting medium with DAPI, and visualised with a Leica DXM-RXA microscope and Q-Fish software.

6.4 Results

ret/PTC-1 chimeric transcripts were detected in eighteen of nineteen (95%) cases of Hashimoto thyroiditis, in addition to the control cell line (TPC-1). Sixteen of these cases (84%) had no evidence of malignancy while the remaining three (16%) displayed concomitant PTC. mRNA extracted from both areas of PTC and Hashimoto thyroiditis showed activated *ret*/PTC-1 in each case. Seven cases of lymphocytic thyroiditis (LT) were examined for expression of the chimeric RET/PTC-1 transcript. Three cases of LT of seven examined had positive signals for *ret*/PTC-1. Of these cases, two had associated PTC. A single case of Grave's disease was analysed, and was negative for RET/PTC-1 expression (Fig.6.1). All cases from HT and LT cohorts harbouring PTC were positive for *ret*/PTC-1 activation (Fig.6.3).

Those cases of HT and LT with PTC were biochemically hypothyroid on presentation. Among the non-specific LT group three were euthyroid on presentation with the remainder hypothyroid, and the case of Grave's disease biochemically hyperthyroid (Table 6.1).

Statistical analysis comparing the incidence of ret/PTC-1 positivity with thyroiditis demonstrated a significant association ($p=0.0104$) (Table 6.2).
Successful amplification of the extracted RNA was achieved in all cases, demonstrated by positive signals for GAPDH.

In-situ TaqMan RT-PCR was used to demonstrate the localisation of the *ret*/PTC-1 transcript intracellularly. The transcript was localised in the cytoplasm of TPC-1 cells. Interestingly, where cells were clustered together in follicle formation the transcript was situated at the luminal edge of the cells, forming a 'ring-like' structure around the lumen of the follicle.

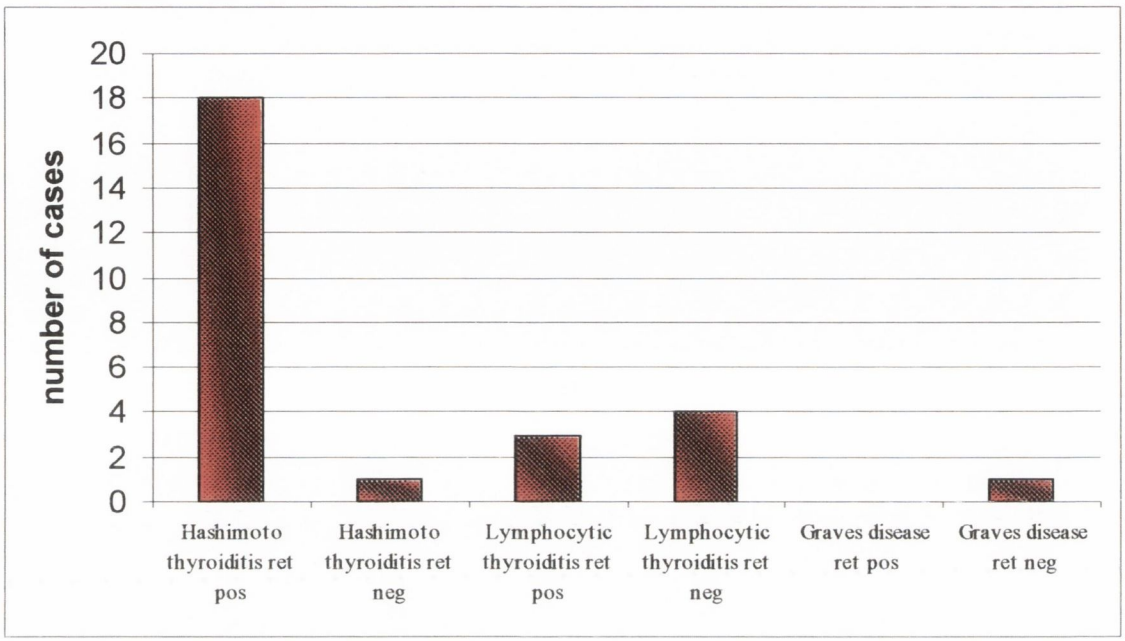
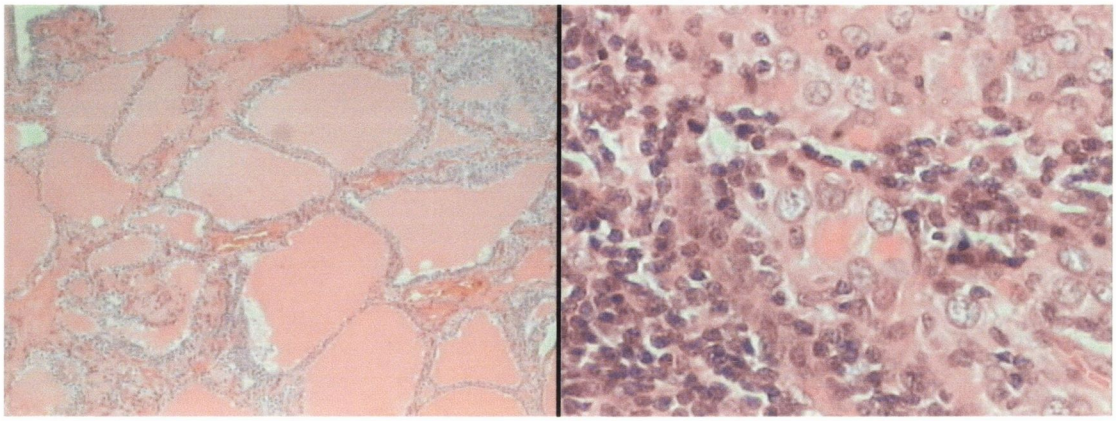
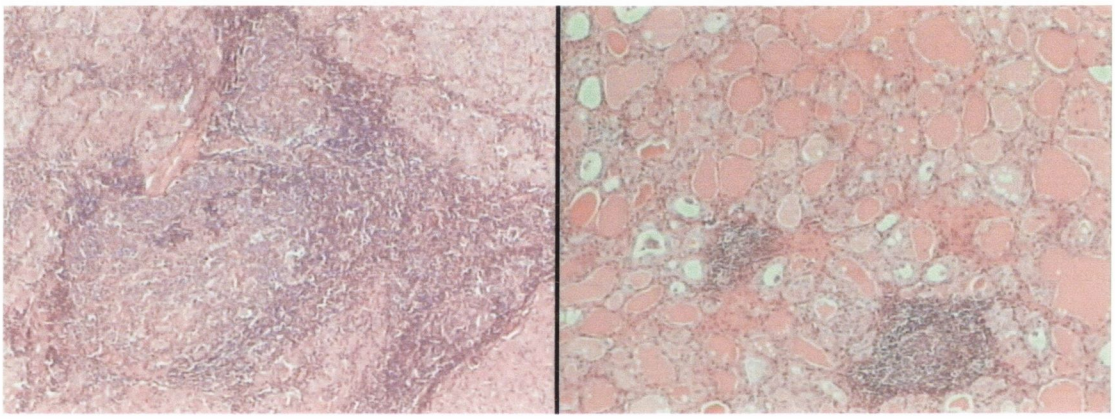


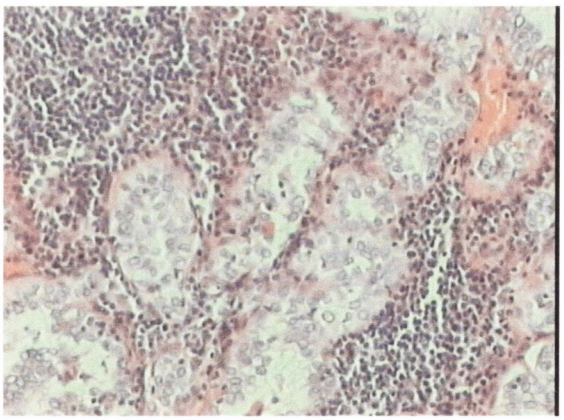
Figure 6.1 RET/PTC-1 expression and associated thyroiditis.



A B



C D



E

A = Grave's Disease - *ret*/PTC-1 negative.

B = Hashimoto Thyroiditis - *ret*/PTC-1 positive.

C = Hashimoto Thyroiditis - *ret*/PTC-1 positive (lower magnification)

D = Lymphocytic Thyroiditis - *ret*/PTC-1 negative.

E = Hashimoto Thyroiditis with concomitant PTC - *ret*/PTC-1 positive.

Figure 6.2 H&E stained sections of thyroiditis

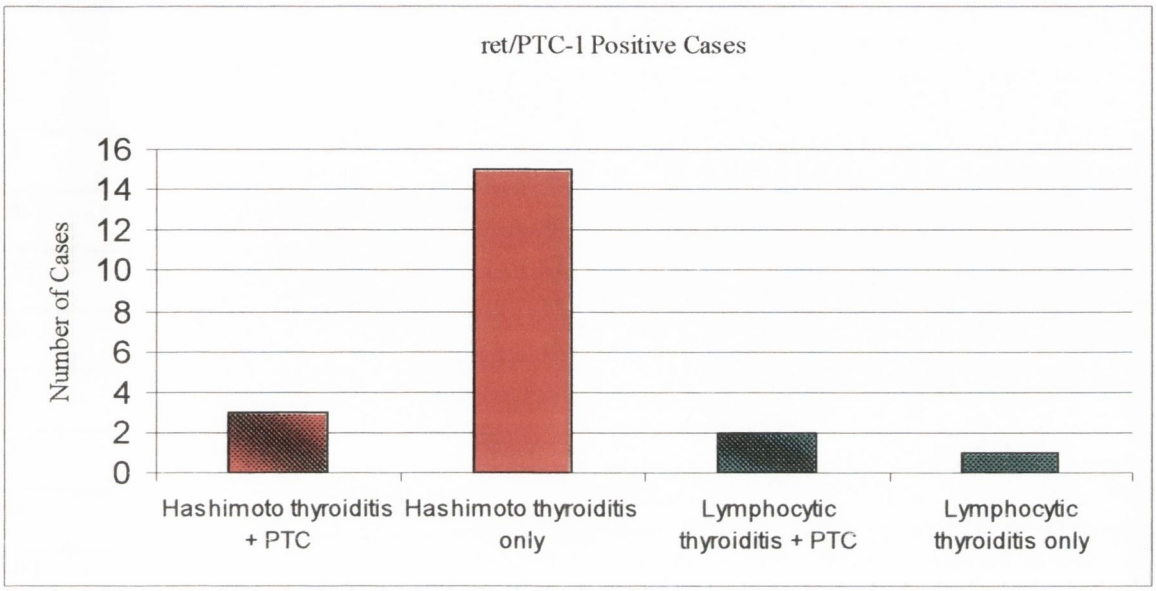


Figure 6.3 Incidence of *ret*/PTC-1 activation and PTC in thyroiditis.

Table 6.1 Diagnosis, Biochemical and *ret*/PTC-1 status.

Case #	Diagnosis	ret/PTC-1 status	Biochemical Status
1	HT	Positive	Hypothyroid
2	HT	Positive	Hypothyroid
3	HT	Negative	Hypothyroid
4	HT	Positive	Hypothyroid
5	HT	Positive	Hypothyroid
6	HT	Positive	Hypothyroid
7	HT	Positive	Hypothyroid
8	HT	Positive	Hypothyroid
9	HT	Positive	Hypothyroid
10	HT	Positive	Hypothyroid
11	HT	Positive	Hypothyroid
12	HT	Positive	Hypothyroid
13	HT	Positive	Hypothyroid
14	HT	Positive	Hypothyroid
15	HT + PTC	Positive	Hypothyroid
16	HT	Positive	Hypothyroid
17	HT	Positive	Hypothyroid
18	HT + PTC	Positive	Hypothyroid
19	LT + PTC	Positive	Hypothyroid
20	HT + PTC	Positive	Hypothyroid
21	LT + PTC	Positive	Hypothyroid
22	LT	Negative	Euthyroid
23	LT	Negative	Euthyroid
24	LT	Negative	Euthyroid
25	Graves Disease	Negative	Hyperthyroid
26	LT	Negative	Hypothyroid
27	LT	Negative	Hypothyroid

HT=Hashimoto thyroiditis

LT=lymphocytic thyroiditis

PTC=papillary thyroid carcinoma

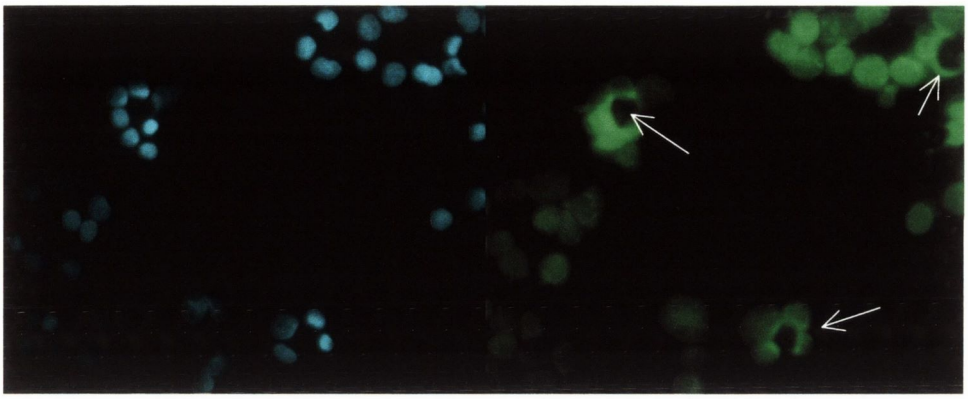
	ret/PTC-1 positive	*ret*/PTC-1 negative
Hashimoto thyroiditis	18	1
Lymphocytic thyroiditis	3	4

Fisher's Exact Test

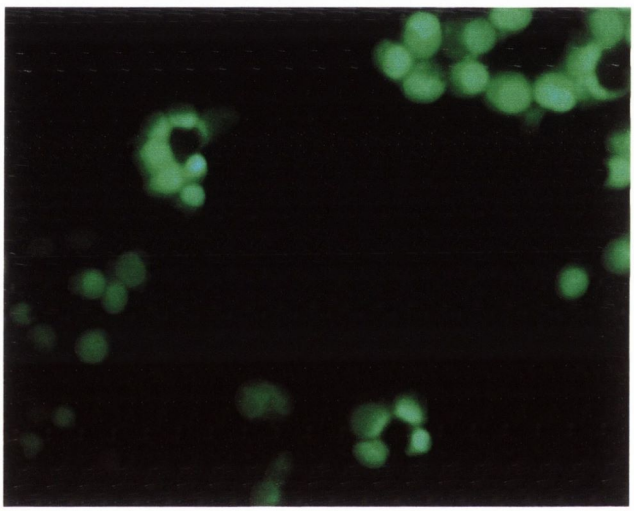
The two-sided p value is 0.0104.

There is a significant association between rows and columns.

Table 6.2 Statistical Analysis of Results



A B



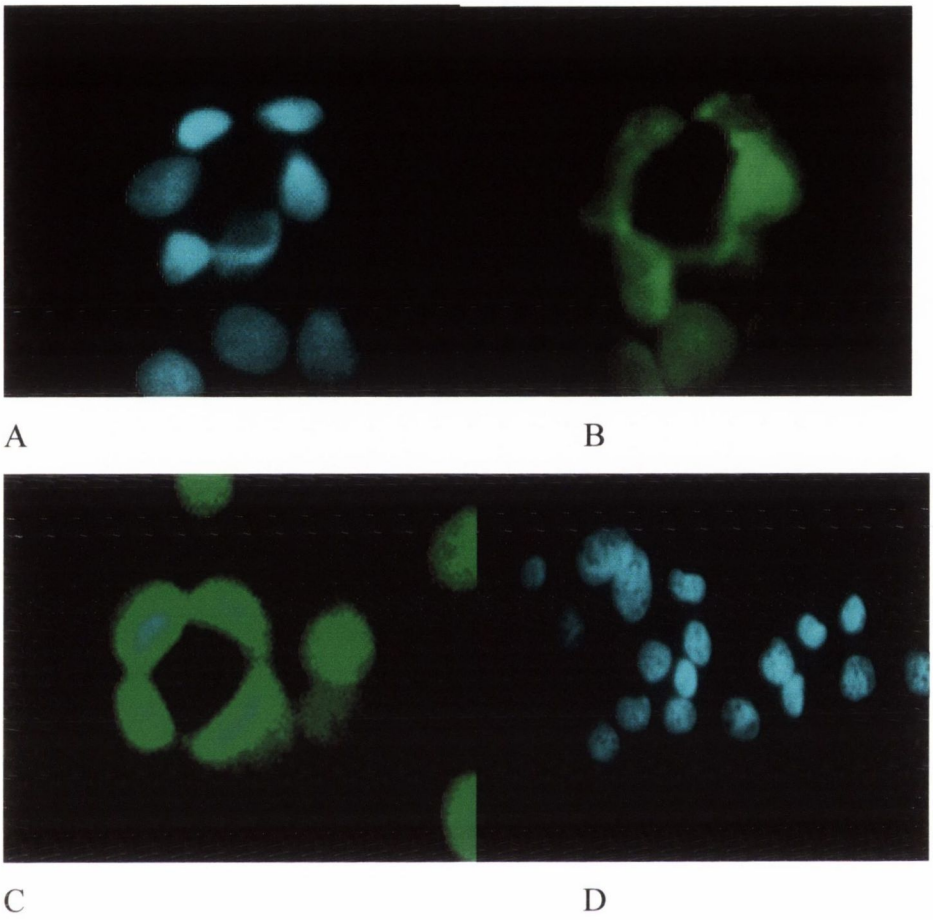
C

A = TPC-1 cells counterstained with DAPI (blue)

B = IS TaqMan RT-PCR for *ret*/PTC-1 in TPC-1 cells (Reporter signal = FITC (green)). Note the localisation of transcript to the 'luminal' surface of follicular cells (arrowed).

C = Combined image of DAPI and FITC stained cells.

Figure 6.4 In situ TaqMan RT-PCR.



A B

C D

IS TaqMan RT-PCR for *ret*/PTC-1 in TPC-1 cell line. (25 cycles 4.5mM Mn^{2+})

A = Nuclear counterstain only (DAPI – blue).

B= TaqMan Reporter signal for chimeric transcript (FITC-green). Transcript is concentrated around the luminal surface of the follicle.

C = Combined image of DAPI and FITC.

D = Negative control (no probe @ 25 cycles).

Figure 6.5 In situ TaqMan RT-PCR.

6.5 Discussion

The *ret*/PTC oncogene, an activated form of *c-ret* has been found as a specific phenomenon in some papillary thyroid carcinomas (PTC) (Jhiang 1994). In a recent study, our group demonstrated a statistically significant incidence of *ret*/PTC-1 activation with concomitant thyroiditis in PTC (Sheils). These findings prompted us to examine a series of tissues from various forms of lymphoid infiltration of the thyroid, among which we detected the *ret*/PTC-1 chimeric transcript in 78%.

The activation of the PTC oncogene has previously been thought to be restricted to PTC (Santoro 1992), although there are varying reports as to whether it may be associated with indolent (Santoro 1992) or aggressive (Sugg 1996) phenotypes of the disease. The detection of *ret*/PTC-1 in 94% of patients with Hashimoto thyroiditis without evidence of malignancy was unexpected and poses a number of interesting questions:

(a) It is possible that *ret*/PTC-1 is a marker for follicular cell activation rather than being linked with the specific development of PTC. However, follicular adenomas and other benign lesions of the thyroid have not been shown to express RET/PTC-1.

(b) Hashimoto thyroiditis may represent an early malignant (pre-microscopic) state to which a sub-set of the population are able to mount an immune response, curtailing the development of PTC, albeit with the auto-immune destruction of thyroid tissue.

Among lymphocytic thyroiditis cases examined, 43% (3/7) demonstrated activated *ret*/PTC-1. There was concomitant PTC in 66% (2/3) of the positive cases, which may account for their positivity.

H4 is the other affected gene in the generation of the *ret*/PTC-1 chimeric transcript. Studies have suggested that H4 codes for a cytoskeletal protein having structural homology with actin (Yap 1997). In a previous study (Sheils in press), our group has demonstrated a failure to express H4 when the *ret*/PTC-1 transcript

is present. It has been suggested that *ret*/PTC-1 activation may influence the growth pattern in PTC (Yap 1997, Fischer 1998). The altered growth pattern may be due to failure of expression of the cytoskeletal protein H4 leading to altered cellular structure or, secondly, because RET/PTC-1 is constitutively phosphorylated.

The findings of in-situ TaqMan RT-PCR on TPC-1 cells where the transcript has been detected in PTC cells support this theory. The localisation of the transcript to the luminal margin of follicular clusters of cells in extremely marked, and implies a level of intercellular communication or architectural co-operation in the formation of these follicles. Further work needs to be done to evaluate the exact nature of the positioning of the *ret*/PTC-1 transcript, however, it seems plausible that it is involved in the morphogenesis of PTC.

Yap et al (1997) have shown that increased tyrosine phosphorylation alters thyroid epithelial organisation in culture by altering the assembly of actin-associated adhesive junctions. They have shown that under the influence of tyrosine phosphorylation, thyroid cells lose their capacity to form follicles and spread and migrate into confluent monolayers. It is possible therefore, that similar cytomorphological alterations occur in-vivo in association with *ret*/PTC-1 activation and may contribute at least in part to the initiation of the auto-immune response characteristic of Hashimoto thyroiditis.

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CHAPTER 7

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE WORK.

7.1 Review of results

This study has involved wide ranging methodology in the search for prognostic markers in thyroid carcinoma. The body of work commenced with conventional histological evaluation of a number of markers in a small series of thyroid neoplasms, including PTC, FTC, MTC FA and ATC. The aim behind this pilot study was to assess to efficacy of currently used biological markers to identify more aggressive tumours.

Manual counting of mitotic and apoptotic bodies is time consuming and subjective, and could not be considered by itself to be an objective prognostic indicator. Further information regarding the proliferative activity of a given tumour may be obtained by combining these counts with immunohistochemical evaluation of markers such as Ki-67. The ATC cohort in this study had significantly higher Ki-67 counts than the control group. However, it would be of more importance to identify those tumours among the well-differentiated category which were likely to progress to more advanced disease. In fact, identifying increased proliferation in the ATC group is akin to closing the stable door after the horse has bolted. The same could be said of p53 detection by immunohistochemistry. There was variation in staining among the ATC group, however, the general lack of staining among the remaining thyroid neoplasias casts a doubt over its usefulness as a prognostic marker.

Similarly, EGFR staining was negative in all but one of the controls, casting doubt over its usefulness in prognostication. It is noteworthy however, that there was a variation in staining pattern among the ATC group, and further work to evaluate the significance of diffuse cytoplasmic or discrete membranous staining patterns is warranted.

Using RT-PCR to evaluate TSHr expression, a range of results was obtained. TSH is the primary hormone regulating thyroid cell function and proliferation. It is reasonable to assume that loss of TSHr in thyroid carcinomas would not only

reduce their responsiveness to TSH but also indicate dedifferentiation of the tumour cells.

The technique of assessment of TSHr expression used in this opening study could not be presumed to be sophisticated. Gel densitometry of amplified bands gives an indication of the level of expression, but the technique is subject to too many variables to provide accurate quantitation.

As evaluation of TSHr expression appeared to yield informative data with regard to prognosis, it was decided to develop a more sophisticated and accurate technique for its evaluation. The TaqMan RT-PCR assay described is a relatively simple technique (provided the instrumentation is available) providing consistent, reproducible results. Because the amplicon size is kept to a minimum, the technique is ideal for use with degraded template, which is the best one can expect from archival material. The generation of a TSHr index was useful in that it normalised the expression data for variations such as differences in degradation due to different fixation, processing or storage conditions. The data obtained using this assay enabled the categorisation of tumours into well- and poorly-differentiated sub-types. In addition, a number of tumours were highlighted whose TSHr index better reflected the activity of the tumour than routine histological grading.

Activation of the *ret* proto-oncogene has been described among papillary thyroid carcinomas (PTC). The most commonly detected variant of *ret* activation is *ret*/PTC-1, accounting for approximately 70% of *ret* activated PTC. Consequently, a TaqMan based RT-PCR assay was designed for its analysis. A number of features were evident from the data obtained in this study.

Firstly, >90% of ATC were shown to harbour the *ret*/PTC-1 chimeric transcript. There is controversy in the literature regarding the association of *ret*/PTC-1 and tumour aggressiveness. Some groups have associated *ret*/PTC-1 activation with indolence in PTC, while others have detected its presence among tumours with distant metastases and disease progression. Our findings support this latter theory.

The variety in findings among different groups may be accounted for by differences in technique and assay design. The TaqMan RT-PCR assay is extremely sensitive and specific, and has been successful in detecting chimeric RNA transcripts where conventional RT-PCR has failed. It would be interesting to evaluate the amount of chimeric transcripts present in ATC versus PTC, to ascertain if *ret* activation is a clonal event in ATC.

The second important observation in the assessment of *ret*/PTC-1 activation among PTC, was the incidence of chronic lymphocytic thyroiditis in association with *ret*/PTC-1 positive tumours. Interestingly those tumours also failed to express H4 (the other gene involved in the chromosomal inversion giving rise to *ret*/PTC-1). H4 codes for the synthesis of a putative cytoskeletal protein. This gave rise to the speculation that *ret*/PTC-1 may influence the growth pattern in PTC, and may also be involved in the initiation of an immunological response similar to that seen in thyroiditis.

It seemed logical, following the observations made in the PTC cohort, to examine a series of non-neoplastic thyroid tissues for *ret*/PTC-1activation. The results of this study provided unexpected data, in that >95% of Hashimoto thyroiditis tissues examined demonstrated activated RET expression. In the majority of cases, this phenomenon occurred with no evidence of associated PTC. The conclusion from this finding was that Hashimoto thyroiditis might represent an early malignant or pre-malignant state, which is detected by the immune system of a subset of the population. These individuals mount an immune response, which curtails the progression to PTC in the majority of instances, albeit with auto-immune destruction of the thyroid gland.

With a view to elucidating the effect of the presence of *ret*/PTC-1 transcripts within the cell a novel in-situ TaqMan assay was established. IS-TaqMan RT-PCR was used to localise the *ret*/PTC-1 transcript in cultured PTC cells. This technique demonstrated that the transcript is indeed cytoplasmic. Further, where cells clustered together in follicle formation the staining pattern was dramatically

different. In this situation the pattern was discrete, and formed a distinct border along the luminal edge of the cells. This data gives further credence to the concept that *ret*/PTC1 is involved in morphogenesis in PTC. Further work in this area is needed to establish the exact role of this chimeric transcript in the development of PTC. In addition, studies on cell adhesion molecules and their expression are justified to determine how the *ret*/PTC-1 transcript is involved in cell-cell signalling, and why its localisation is altered with follicle formation. The fact that genetic rearrangement has occurred at the DNA level in these cells is the first step along a cascade of events culminating in the existence of a morphologically altered cell. Expression of the chimeric RNA transcript leads to the synthesis of a mutant RET/PTC-1 protein with concomitant altered or diminished expression of normal H4 protein. The consequences of these two scenarios are such that internal framework of the cell is disrupted and altered.

In order to formulate a hypothesis on the precise role of genetic rearrangement and *ret* activation in Hashimoto Thyroiditis and subsequently PTC, aspects of a number of theories must be extracted and interlinked. The first deals with tensegrity forces and their effects within the cell, and the second with the concept of danger in the immune system, and how the body deals with it.

7.2 Tensegrity

It seems reasonable to speculate that the forces of tensegrity may be involved with the altered cellular morphology observed in PTC and indeed in other tumour types. Donald Ingber (1998) has postulated the existence of a universal set of building rules which function in the design of organic structures - from simple carbon compounds to complex cells and tissues.

Recently, it has been shown (Ingber 1999) that cells can sense mechanical stresses, through changes in the balance of forces that are transmitted across

transmembrane adhesion receptors that link the cytoskeleton to the extracellular matrix and to other cells (e.g. integrins, cadherins and selectins). The mechanism by which these mechanical signals are transduced and converted into a biochemical response appears to be based on the finding that living cells use a tension dependent form of architecture known as tensegrity, to organise and stabilise their cytoskeleton.

One of biology's most fundamental and complex riddles is how groups of molecules assemble themselves into whole, living organisms. The answer may depend on "tensegrity," a versatile architectural standard in which structures stabilize themselves by balancing forces of internal tension and compression. The same relatively simple mechanical rules, operating at different scales, may govern cell movements, the organization of tissues and organ development.

Over the last decades, scientists have strived to advance our understanding of how the human body works by defining the precise properties of critical molecules like DNA. Understanding the make-up of the component parts of a complex machine, however, does not explain how the whole system works. The phenomenon by which components join together to form larger, stable structures is not easily predicted by the characteristics of their individual parts. An astoundingly wide variety of natural systems, including carbon atoms, water molecules, proteins, viruses, cells, tissues and entire organisms are constructed using a common form of architecture known as tensegrity. This term refers to a system that stabilises itself mechanically because of the way in which tensional and compressive forces are distributed and balanced within the structure. Tensegrity structures are mechanically stable not because of the strength of the individual members but because of the way the entire structure distributes and balances mechanical strength.

Inside the cell, a gossamer network of contractile micro-filaments extends throughout the cell, exerting tension. The tensegrity model suggests that the structure of the cell's cytoskeleton can be changed by altering the balance of

physical forces transmitted across the cell surface. Alterations in the levels of expression of RET/PTC-1 and H4, lead to changes in cytoskeletal geometry and mechanics and thus could affect biochemical reactions and even alter the genes that are activated and thus the proteins that are made.

It has been demonstrated (Singhvi 1994) that simply modifying the shape of a cell, could switch cells between different genetic programmes. This group of researchers demonstrated a greater propensity for division among cells that spread flat in culture, while round cells, which were prevented from spreading displayed increased apoptosis. Cells maintained in an intermediate state became differentiated in a tissue specific manner. Thus mechanical restructuring of the cell or cytoskeleton dictates the cell's activity. This concept further reinforces the potential association between variations in expression of H4 and RET/PTC-1 and the observed morphology in PTC.

Cai et al (1998) have demonstrated that increased myosin light chain phosphorylation correlates with increased cytoskeletal stiffness and further suggested that changing the mechanical characteristics of the cytoskeleton alters intracellular signalling pathways that regulate cell growth and division.

Recent studies (Ingber 1999) confirm that alterations in the cellular force balance can influence intracellular biochemistry within focal adhesion complexes that form the site of integrin binding as well as gene expression in the nucleus, while Eckes et al (1998) have established that alterations in cytoskeletal expression of vimentin render cells impaired in all functions depending upon mechanical stability. These data give further support for the role of *ret* activation in PTC, as H4 possesses considerable homology with actin and vimentin, and loss of expression is bound to affect intracellular mechanical stability, while constitutively phosphorylated ret-TK must affect cellular tensegrity.

7.3 The Danger Theory

The 'Danger Theory' postulated by Polly Matzinger (1994) addresses the question of what causes the immune system to respond, and contradicts the previously held dictum of self versus non-self. This group suggests that danger is sensed by tissues themselves, which signal the professional antigen presenting cells among them. The signals sent by stressed cells are not known but they could be of several types. A dendritic cell might become activated if a cell to which it is connected suddenly dies, simply from the sudden loss of connection. It might have receptors for heat shock protein elaborated by stressed cells or for a protein, normally found only on the inside of intact healthy cells, that leaks out only if the cell dies lytically. There is a myriad of potential ways in which a tissue could communicate danger to its local antigen presenting cells (APCs).

There are three basic laws and one minor exception, which govern the danger theory. The first law, which is taken from the Bretscher-Cohn and Lafferty-Cunningham models, assumes that resting T cells need two signals to be activated; signal 1 from TCR binding to MHC/peptide and signal 2 (co-stimulation) from an APC. It states that T cells die if they receive signal 1 without signal 2, and become activated if they receive both.

The second law states that resting T cells can only receive co-stimulatory signals from APCs. Interdigitating dendritic cells (and macrophages) can serve as APCs for both naïve and experienced T cells. B cells can re-stimulate experienced but not naïve T cells. Other tissues may express surface MHC/peptide complexes, however, they do not express the necessary co-stimulatory signals needed by T cells for activation.

The third law states that the activated effector stage persists for a limited period of time. During this time, T cells do not require co-stimulatory signals and can be triggered to function (e.g. assist B cells or kill targets) by signal 1 alone. After this

stage they either die or return to a resting state from which they can only be drawn again by the appropriate combination of signal 1 and signal 2.

The conclusion of this theory is that each tissue essentially has three functions. First it does its normal job. Second, by elaborating stress signals, it alerts the rest of the immune system to the presence of danger. Third by displaying its own antigens in the absence of co-stimulation, it induces tolerance to itself.

The necessary exception to these laws lies in the thymus, and is designed to delete those T cells, which are capable of recognising dendritic cells. Thymocytes pass through a developmental stage during which they are 'tolerisable but not yet activatable'. During this stage the pathways for receipt of second signals are not yet hooked up. Hence, at this stage the thymocyte would be unable to receive second signals from any cell including APCs. Receiving signal 1 without signal 2 would thus eliminate any thymocye recognising the normal surface MHC/antigen profile of a dendritic cell.

Mature T cells stimulated by signal 1 and signal 2 from activated APCs (alerted to danger by distressed cells), can multiply, become effector cells and circulate in response to the danger. Each killer destroys a few distressed cells and then reverts to a resting state and drains back to a local lymph node, where if danger signals are still being received it will be re-activated. This process will continue until there are no more APCs presenting the target antigen. A select number of experienced cells will re-circulate as resting memory cells awaiting their next encounter.

An important point to stress at this point is that killer cells induce apoptosis in their targets. Thus, death induced by killer cells does not signal APCs to perpetuate the immune response. Only real danger can do that.

The danger theory also has an interesting outlook on tumours. It states that there is no intrinsic difference between a rapidly dividing tumour cell and for example a

rapidly dividing haematopoietic cell or gut cell. If it dies, it does so via apoptosis. Thus, there is no particular reason the immune system should distinguish it from any other rapidly dividing cell type unless it becomes stressed or necrotic. In addition, tumour-infiltrating lymphocytes only work under certain conditions. If TILs are experimentally removed from a tumour, activated and re-injected along with a source of IL-2 they will return to the tumour and engage in a round of destruction. If the tumour burden is small this may be sufficient to eradicate it. If however, the tumour cell load is too great, the T cells will go through their natural cycle of resting down and waiting to be re-stimulated. Without a source of activated APCs they will remain in a resting state to be tolerised by recognition of tumour antigens in the absence of signal 2.

The danger theory advocates an immune system in which the availability of second signals governs immunity and tolerance, an immune system, which is a highly interactive network, acting upon input from all bodily tissues. It is a flexible system that changes as the organism changes, and is in harmony with both its internal and external environment.

7.4 The role of tensegrity and danger in relation to ret activation.

If we consider DNA to be a fundamental component in the architecture of the cell, which in all individuals has some areas of inherent structural weakness, then external insult by an agent such as viral infection or ionising radiation could, by altering the tensegrity forces within the nucleus bring about a translocation such as is seen in *ret* activated follicular thyroid cells. This change need not of itself alert the immune system to potential danger, however, it does represent an unbalanced state within the cell. When this chimeric gene is transcribed, a previously unexpressed protein is generated. The fusion protein (*ret*/PTC-1) is unusual in that it has components of a tyrosine kinase molecule (which is now constitutively

phosphorylated) and a cytoskeletal protein (H4). In addition the cell has lost the capacity to express H4.

Bearing these facts in mind, it seems reasonable to assume that such a cell is subject to considerable alteration in its tensegrity forces. Alterations in tensegrity have been demonstrated to have significant downstream effects with regard to cell shape, growth, adhesion properties and even biochemical function. Therefore it is plausible that a *ret*/PTC-1 activated cell could signal danger to the immune system of the affected host. If the dendritic cells of the thyroid recognise this unstable cell due to its altered tensegrity, they could elicit a response as described in the danger theory.

Over the next relatively short period, Hashimoto Thyroiditis would ensue provided the immune response was swift and effective enough, with activated T cells descending on the *ret* activated cells in response to the danger signals they emit. With time, if the *ret* activated cells were not completely eradicated, they could stabilise somewhat. In this situation the tensegrity forces within the cell would realign themselves to accommodate the new state of affairs, and establish a new balance. These cells would no longer be under stress and would not pose a danger threat to the immune system. Unchallenged by immune attack, the mutant cells could grow as dictated by their new balance of tensegrity. Singhvi has already demonstrated in his work that whether cells grow flat or clustered on top of one another has profound implications on whether the tissue proliferates, dies or differentiates. As a consequence of the localisation of the chimeric transcript to the apical edge of follicular cells, these cells would grow with a different morphology to normal follicular cells of the thyroid, culminating ultimately in papillary thyroid carcinoma.

If this theory holds true, it has wide ranging implications for not only thyroid carcinoma but also raises questions such as what role (if any) does ret or H4 have in the development of papillary ovarian cancer. The morphology is similar in

papillary ovarian cancer to that seen in PTC, and it would be interesting to establish if similar molecular alterations are involved in this tumour type.

As suggested by early 20th-century Scottish zoologist D'Arcy W. Thompson, who quoted Galileo, who in turn cited Plato: the Book of Nature may indeed be written in the characters of geometry.

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