STUDY OF SOME GENE ALTERATIONS IN THE INITIATION AND PROGRESSION OF PAPILLARY CARCINOMA OF THE THYROID.

ESTUDO DAS ALTERAÇÕES DE ALGUNS GENES NA INICIAÇÃO E PROGRESSÃO DO CARCINOMA PAPILAR DA TIREOIDE.

ANA PAULA SOARES DIAS FERREIRA

PORTO
1998

STUDY OF SOME GENE ALTERATIONS IN THE INITIATION AND PROGRESSION OF PAPILLARY CARCINOMA OF THE THYROID.

ESTUDO DAS ALTERAÇÕES DE ALGUNS GENES NA INICIAÇÃO E PROGRESSÃO DO CARCINOMA PAPILAR DA TIREOIDE.

ANA PAULA SOARES DIAS FERREIRA

PORTO, 1998

DISSERTAÇÃO DE CANDIDATURA AO GRAU DE DOUTOR APRESENTADA À FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO.

Artigo 48º, § 3º - A Faculdade não responde pelas doutrinas expendidas na dissertação. (Regulamento da Faculdade de Medicina do Porto - Decreto-Lei nº 19 337, de 29 de Janeiro de 1931).

Ao abrigo do Art. 8º do Decreto-Lei nº 388/70 fazem parte integrante desta dissertação os seguintes trabalhos já publicados ou em publicação.

Soares P, Sobrinho-Simões M: Proliferative activity of human thyroid tumors evaluated by proliferating cell nuclear antigen/cyclin. Immunohistochemical studies. (Letter-to-the-editor). **Cancer** 73: 2879-2880, 1994.

Soares P, Sambade C, Sobrinho-Simões M: Expression of C-erb B2 in tumours and tumour-like lesions of the thyroid. **International Journal of Cancer** 56:459-461, 1994.

Soares P, Cameselle-Teijeiro J, Sobrinho-Simões, M: Immunohistochemical detection of p53 in differentiated, poorly differentiated and undifferentiated carcinomas of the thyroid. **Histopathology** 24:205-210, 1994.

Soares P, dos Santos NR, Seruca R, Lothe RA, Sobrinho-Simões M: Benign and malignant thyroid lesions show instability at microsatellite loci. **European Journal of Cancer** 33:293-296, 1997.

Soares P, Berx G, van Roy F, Sobrinho-Simões M: E-cadherin gene alterations are rare events in thyroid tumors. **International Journal of Cancer** 70:32-38, 1997.

Soares P, Fonseca E, Wynford-Thomas D, Sobrinho-Simões M: Sporadic ret-rearranged papillary carcinoma of the thyroid: A subset of slow growing, less aggressive thyroid neoplasms? **Journal of Pathology** (em publicação).

Em cumprimento do disposto no referido Decreto-lei declara que participou activamente na recolha e estudo do material incluído em todos os trabalhos, tendo redigido os textos com a activa colaboração dos outros autores. Inclui também resultados, não publicados, relativos à expressão de uPAR.

Agradecimentos

Há alturas em que as palavras nos parecem demasiado curtas para o que queremos transmitir.

Devo ao Professor Sobrinho-Simões a oportunidade e o estímulo para tudo o que concretizei profissionalmente. Um Obrigado muito sentido pelo apoio científico, pedagógico e humano que me deu ao longo deste percurso.

My gratitude to Professor Wynford-Thomas, my co-supervisor, for all the support he gave to me during these years. I thank also to Professor Wynford-Thomas and his group for having given me the chance to work and learn with them.

Agradeço ao Professor Daniel Serrão a disponibilização de meios para concretização de parte dos trabalhos apresentados e o incentivo que sempre me dispensou.

Aos co-autores das publicações agradeço a sua colaboração.

Agradeço à amiga Raquel Seruca que me iniciou nos meandros da biologia molecular.

À Fátima Magalhães quero agradecer a disponibilidade profissional e a disponibilidade da sua amizade.

À Paula e à Dina quero agradecer a colaboração nos trabalhos e a solidariedade pessoal.

Agradeço à Prof. Leonor David a sua acolhedora e estimulante ajuda.

Agradeço ao Eng. Marta, uma das "vítimas" do meu stress (informático).

I am grateful to Professor Van Roy and to Geert Berx for their collaborative attitude and also for their hospitality during my stay at Ghent.

It was nice to work for a while at Groningen, thank you Robert.

São já muitas as "gerações" de colegas e amigos no IPATIMUP aos quais estou grata pelo seu apoio e carinho. Dos "seniores" aos "juniores" um abrangente agradecimento.

Agradeço à JNICT e ao Programa PRAXIS XXI pelas bolsas concedidas.

Um agradecimento em jeito de dedicatória à Tati e ao Tomané.

NOTA EXPLICATIVA

A presente Dissertação está escrita em inglês na sua quase totalidade, exceptuando o Sumário e Conclusões, pelo facto de o Doutor Wynford-Thomas ter sido o seu co-orientador.

TABLE OF CONTENTS

ABBREVIATIONS

ATP - Adenosine triphosphate

cAMP - ciclic-Adenosine monophosphate

CDGE - Constant denaturating gel electrophoresis

CDK2 - Cyclin dependent kinase

DAG - Diacylglycerol

DNA - Deoxyribonucleic acid

EGF - Epidermal growth factor

EGFR - Epidermal growth factor receptor

erbB2 - Avian erythroblastosis virus B2

FMTC - Familial medullary thyroid carcinoma

GAP - ras GTPase activating protein

GDNF - Glial-cell-line-derived neurotrophic factor

GDP - Guanosine diphosphate

GRB2 - Growth factor binding protein 2

GTP - Guanosine triphosphate

HGF - Hepatocyte growth factor

HGFR - Hepatocyte growth factor receptor

hMSH2 - human Mut S homologue 2

HPVE6 - Human papilloma virus protein E6

HPVE7 - Human papilloma virus protein E7

IGF-I- Insulin-like growth factor I

IGF-IR- Insulin-like growth factor I receptor

IP3 - Inositol triphosphate

IRS-1 - Insulin Receptor Substrate-1

MAP - Mitogen activated protein

MEK - Map/Extracellular-signal-regulated kinase

MEN2A - Multiple endocrine neoplasia 2A

MEN2B - Multiple endocrine neoplasia 2B

MI - Microsatellite instability

mRNA - messenger Ribonucleic acid

NTN - Neurturin

PAI-1 - Plasminogen Activator Inhibitor

PAX-8 - Paired box gene-8

PCNA - Proliferating cell nuclear antigen

PCR - Polymerase chain reaction

PIP2 - Phosphatidylinositol 4,5-diphosphate

PKC - Protein kinase C

PLC - Phospholipase C

PTC - Papillary thyroid carcinoma

RB - Retinoblastoma susceptibility gene

RET - Rearranged during transfection

RNA - Ribonucleic acid

SH2 - src homology 2

SOS - Son-of-sevenless

SSCP - Single strand conformation polymorphism

T3 - Triiodothyronines

T4 - Tetraiodothyronines

TGFα - Transforming growth factor α

TGFβ - Transforming growth factor β

TGFβR - Transforming growth factor β receptor

TKR - Tyrosine kinase receptors

TPO - Thyroid-peroxidase

TRH - Thyrotropin-releasing hormone

TSH - Thyroid-stimulating hormone

TSHR- Thyroid-stimulating hormone receptor

TTF-1 - Thyroid transcription factor-1

TTF-2 - Thyroid transcription factor-2

uPA - urokinase type - Plasminogen Activator

uPAR - urokinase type - Plasminogen Activator Receptor

WHO- World Health Organization

INTRODUCTION

Foreword

Thyroid cancer being the most frequent type of endocrine neoplasia presents features that make it an attractive model for the study of oncogenesis.

Using epidemiologic and clinic evidence it has been firmly established that thyroid cancer is often a slow growing neoplasia, carrying high survival rates and long disease free intervals; thyroid carcinoma is more frequent in females and though it may arise at any age, it is more frequent after the age of forty.

Despite the aforementioned general pattern, thyroid cancer shows a wide spectrum of histotypes comprising well differentiated (follicular and papillary) carcinomas usually carrying a very good prognosis in one end of the spectrum and one of the most aggressive forms of human tumour, the undifferentiated carcinoma, in the other end. There is evidence pointing to the existence of a multistep evolution from better differentiated to less differentiated histotypes and a model of tumour progression was thus proposed (224).

Based in the WHO Classification (81), the main histologic types of thyroid carcinoma can be characterized as follows:

Differentiated carcinomas

Papillary thyroid carcinoma (PTC) - A malignant epithelial tumour showing evidence of follicular cell differentiation, typically displaying papillary and follicular structures and characteristic nuclear features. Most PTC contain complex branching papillae that have a fibrovascular core covered by a single layer of tumour cells. The nuclei of PTC usually display a "ground glass" appearance, large size, irregular contour, prominent grooving and pseudo-inclusions resulting from cytoplasmic invaginations. These nuclear features namely the large size, the grooving and the pseudo-inclusions are very characteristic and have been used to establish on firm grounds the cytological diagnosis of PTC. Psammoma bodies occur in almost half of papillary carcinomas and

practically never in other thyroid lesions. Follicles are almost always present and may be the predominant component. Multiple microscopic tumour foci distant from the primary tumour including the contralateral lobe of the thyroid are often seen and in most cases are thought to represent intraglandular spread of the carcinomas; however, the possibility of multicentricity can not be ruled out. Regional lymph node metastases are extremely frequent in PTC regardless of the size of primary tumours whereas lung metastases are relatively rare except in some subtypes of PTC (81).

Follicular thyroid carcinoma (FTC) - A malignant epithelial tumour showing evidence of follicular cell differentiation but lacking the diagnostic features of PTC. At variance with the latter, FTC usually display a well formed capsule. The structure of follicular carcinoma is extremely variable ranging from well formed follicles containing colloid to a solid, cellular growth pattern. Follicular carcinomas are classified according to their degree of invasiveness as minimally invasive ("encapsulated") and widely invasive. Lymph node metastases are uncommon in follicular carcinomas. Distant metastases are commonly associated with widely invasive tumours being the lungs and bones the most frequent metastatic sites (81).

Medullary thyroid carcinoma - A malignant tumour showing evidence of C-cell differentiation and calcitonin production (81) that will not be addressed in this work.

Undifferentiated thyroid carcinoma - A highly malignant tumour composed in part or wholly of undifferentiated cells. The tumour is typically composed of varying proportions of spindle, polygonal and giant cells, and is considered to represent the terminal stage of the dedifferentiation of a prexisting follicular or papillary carcinoma due to the presence in many undifferentiated carcinomas of a residual well differentiated component (81).

A substantial part of our work has been focused in PTC, not only because it is the most frequent type of thyroid neoplasia, but also because it represents a particularly original

model in terms of oncobiology. The most interesting clinicopathologic features of PTC will be summarized in the following paragraphs.

PTC represents 70% of the clinical evident thyroid carcinomas (27, 80, 121), but this figure is probably underestimated since in addition to the clinically evident PTC there are many small PTC that will be progressively more and more often detected in the future due to the ever increasing utilization of sophisticated imagiology procedures (It has been reported a 3-36% prevalence at autopsy of the so-called "occult" or "papillary microcarcinoma") (58, 75, 108, 166, 182). Papillary microcarcinoma of the thyroid, i.e., papillary carcinomas with a diameter of 1cm or less are indeed extremely common and it has been suggested that they rarely progress to clinical evident cancers (it is not known if they arise and "freeze" as microcarcinomas or if they may even regress). Despite their tiny size, microcarcinomas are able to metastasize, and the first clinical manifestation of its presence may be the finding of metastatic tumours in the regional lymph nodes. The frequency of lymph node metastases in papillary microcarcinomas is very high in most of the series on record (22, 58), thus supporting the assumption that papillary microcarcinomas "epitomise" the dissociation between proliferation/growth and metastasis.

No matter the size of the tumours, PTC frequently involve cervical lymph nodes at diagnosis (37%-65%) (22, 58, 80, 122). At variance with the majority of the tumours arising in other organs, no, or only marginal prognostic significance, has been associated with the presence of nodal metastases in PTC. Oversimplifying the issue one may advance that in most series cervical lymph node metastases appear to be linked to tumour recurrence and disease free interval but not with the overall survival of the patients (58, 80, 122, 131).

Nodal involvement has been reported to be more frequent in young patients (22, 58, 122) but this fact does not imply a worse outcome. The presence of distant metastases, which has a bimodal pattern, being more frequent before 20y and after 60y (122), has a

clearly different prognostic significance in these two groups: relatively "benign" in young patients and extremely lethal in elderly patients. Age at diagnosis is indeed one of the strongest and most consistent prognostic factor in terms of survival/mortality of patients with PTC. Part of the aforementioned prognostic variability stems from differences in the therapeutic response to radioactive iodine which tends to be very good in PTC of young patients and regular or bad in PTC of elderly patients.

PTC mainly metastasize via lymphatic vessels to regional lymph nodes in contrast to FTC which mainly metastasize via blood vessels to distant organs. Distant metastases are thus more frequent in (widely invasive) FTC than in PTC both at diagnosis and at neoplastic recurrences. The biologic aspects underneath the diverse metastatic pattern of the two types of differentiated thyroid carcinoma are not yet known but it is interesting to realize that despite easy spreading to regional lymph nodes, common PTC usually lacks the capacity to invade and colonize distant organs. At variance with this "general rule" some rare morphologic variants of PTC show a high propensity to metastatizise to distant organs, the lungs being the most frequently involved site, as is the case of the diffuse follicular variant of PTC and the diffuse sclerosing variant of PTC, both involving diffusely one or both lobes of the thyroid and occurring usually in young patients (58, 60).

PTC are often associated with prominent extracellular matrix response (desmoplasia) and frequently display intense lymphocytic infiltration. In some PTC the fibroblastic/myofibroblastic response to the growth of neoplastic cells is more prominent than the deposition of collageneous and/or hyaline extracellular matrix. This feature is particularly obvious in some rare subsets of PTC (PTC e.g. with fasciitis-like stroma) as well as in the advancing edge of many PTC with an infiltrative growth pattern (As a rule of thumb one may say that excluding the so-called encapsulated variant of PTC, usually composed of follicles and/or trabeculae, most PTC are clearly infiltrative tumours in contrast to the pushing pattern of growth of almost every FTC). It is tempting to relate this infiltrative "nature" of most PTC, regardless of the prominence

of the stromal response and lymphocytic infiltration, with the tendency of PTC to disseminate throughout the thyroid gland and to give rise to regional lymph node metastases (22,58).

Some of these "peculiarities" of PTC were "in the back of our mind" in the studies we have performed within the context of this PhD Project.

We will now review briefly the basic data on the regulation of thyroid function and growth, ending with the comments on the available data on PTC oncogenesis we regard as the most important background information for our own study.

Development, anatomy and histology of thyroid gland

The thyroid gland develops as an endodermic diverticulum in the middle of the floor of the primitive pharynx at the level of the first pharyngeal pouch. The ultimobranchial body, originated from the fifth pouch, becomes incorporated into the developing thyroid, and it is thought that it is the source of at least some of the parafollicular cells. The first follicles with colloid arise at 12 weeks. The fully developed thyroid is a highly vascular bilobed gland, the two lobes being connected together by a thin band of tissue called the isthmus. It lies over the ventral aspect of the trachea to which it is attached by connective tissue and weighs approximately 20 grams (111).

Histologically, the functional units of the thyroid gland are the thyroid follicles, irregular spheroidal structures composed of a single layer of cuboidal epithelial cells bounded by a basement membrane. The follicles (estimated number: $2\text{-}3x10^7$ in the adult) are variable in size and contain a gel-like material termed colloid. The cells are polarized towards the lumen filled with colloid. Each follicle is enveloped by a thin basal lamina, a delicate network of reticular fibers and a plexus of capillaries. The follicular cells have a round or ovoid nucleus, poor in heterochromatin, and containing one or two nucleoli (53).

In actively secreting thyroid glands, the follicles tend to be small and the amount of colloid diminished; the cuboidal lining cells are relatively tall reflecting active hormone synthesis and secretion. Conversely, the follicles of less active thyroid glands are distended by stored colloid and the lining cells appear flattened against the follicular basement membrane. The thyroid is unique in having a histological organisation that provides for extracellular storage of its product in the lumen of the follicles (18).

A second secretory cell type - C-cells or parafollicular cells - is found in the thyroid gland either as single cells scattered among the follicular cells or as small clumps in the interfollicular spaces. Parafollicular cells synthesise and secrete calcitonin in direct response to raised blood calcium levels (18).

Thyroid function

The main function of thyroid is to concentrate iodide and to perform the biosynthesis of thyroid hormones which are essential for the growth and development and for the maintenance of the level of metabolic activity in the organism.

Thyroid hormones - synthesis and release

The biosynthesis of thyroid hormones occurs in three sequential steps: trapping of circulating iodide in follicular cells; binding of iodide to tyrosine residues in the thyroglobulin molecule and coupling of mono- and diiodotyrosines into tri- and tetraiodotyronines. The concentration of iodide in the follicular cells – "iodide trapping" – is achieved through an active pump mechanism at the basal plasma membrane, which uses a sodium mediated Na+ K+ ATPase-like system. This highly efficient mechanism of "iodide trapping" usually achieves an intracellular iodide concentration 25 times greater than the plasma concentration. Once inside the cell, iodide is rapidly oxidized by TPO, prompting iodide binding to amino acids, mostly to tyrosine amino acids in the thyroglobulin glycoprotein. The iodination of tyrosine residues occurs in one or two positions, originating mono- and diiodotyrosine groups on the thyroglobulin molecule. Coupling of mono- and diiodotyrosine groups originate tri- and tetraiodotyronine groups

within one chain or between two chains of thyroglobulin. All the iodination/coupling process occurs by the action of TPO at the follicular cell-colloid interface before secretion of thyroglobulin into follicular spaces. The thyroglobulin molecules containing the iodothyronines are stored in the follicles as colloid and endocytosed by follicular cells under TSH stimulation. Once within the cells the colloid droplets fuse with lysosomes and the thyroglobulin molecules are degraded originating cleavage products of iodotyrosine and iodotyronine that go into the cytoplasm. Mono- and diiodotyrosines are rapidly deiodinated and the tyrosil and iodide residues can then be re-utilized. Tri- and tetraiodothyronine cleavage products constitute the T3 and thyroxine thyroid hormones and are released into the blood (17, 111).

Control of thyroid function

The primary control of thyroid function is mediated by TSH a glycoprotein secreted by the anterior pituitary in response to TRH. TRH is a tripeptide secreted by cells in the hypothalamus, and carried by the hypothalamohypophyseal portal system to the anterior pituitary where it binds to specific receptors on the thyrotroph cells, stimulating their release of TSH. The ability of these cells to respond to TRH is under the feedback control of T3 and T4. An excess of circulating thyroid hormones diminishes the response of the thyrotroph cell to TRH and a deficiency of those hormones increases their response. TSH binds to receptors on the basolateral domain of the follicular cell membrane and the cells rapidly respond with increased intracellular cAMP concentration, accelerated iodine uptake and hormone synthesis (17, 53).

Cell signalling and transduction mechanisms in the follicular cell

Function and growth are tightly regulated in cells by cell signalling molecules (hormones and hormone-like molecules), their receptors, their pathways of signal transduction and second messengers, establishing a communication network. We will briefly review some aspects of this network in thyroid follicular cells.

G-protein linked receptors

The characteristic actions of a hormone depend on its recognition of (and by) the target cell at the level of receptor molecules. In cell membrane receptors the hormone first binds to its receptor to form a complex which subsequently interacts with various nucleotide regulatory proteins within the membrane. Receptors in which the regulatory molecules are guanosine nucleotide proteins are called G-protein linked receptors and the TSH receptor belongs to this class (17).

TSH receptor presents a serpentine membrane spanning structure comprising a single polypeptide chain which crosses the lipid bilayer of the plasma membrane seven times. Three hydrophilic loops project from the extracellular cell surface into the cytosol. Binding of the hormone to the extracellular regions, leads to conformational changes in the intracellular C-terminal domain and to the interaction with G-protein embedded in the fluid membrane. G-proteins are a family of at least four subgroups of proteins which act as transducers of hormone activation by modulating the activity of several critical enzymes (e.g. adenylate cyclase and phospholipase C), known as catalytic units, and alteration of ion chanels for Ca^{2+} and K^{+} within the membrane structures (17, 29, 35).

Each G-protein is composed of three subunits α, β and γ. The β and γ subunits are common to different G-proteins and can combine with four different types of α subunits (s, q, i and o). The chief G-protein that transduces signals from TSH is a trimeric stimulatory G-protein with a $G_s\alpha$ subunit, but $G_q\alpha$ activations can also take place (42). In its resting state, $G_s\alpha$ is bound to a nucleotide diphosphate, GDP and complexed with β and γ subunits. Binding of an agonist to the receptor changes its conformation,

causing subsequent association of the hormone-receptor-G-protein complex leading to displacement of GDP by GTP, which results in dissociation of the activated G-protein from the receptor. When activated, the subunits dissociate and the α subunit binds and activates the catalytic unit. The catalytic units (also referred as effector proteins) are themselves protein enzymes. In normal circumstances, the system is reversible, since the α subunit is capable of exhibiting guanosine triphosphatase (GTPase) activity, thereby hydrolysing the bound GTP to GDP (29, 35, 198).

The second messengers compose a system that mediates the various intracellular effects which characterise the actions of the hormone. Two different types of enzymes, adenylate cyclase and phospholipase C, are important for the mediation of the actions of hormones *via* G-proteins. They provide separate routes by which peptide hormone action at the cell surface may be transmitted within the cell.

Gsα subunit activates adenylate cyclase which in the presence of magnesium or manganese ions induces the formation of the cyclic nucleotide c-AMP from ATP. c-AMP can then activate specific cytoplasmic protein kinases (c-AMP-dependent protein kinases), by binding to their regulatory subunits. These activated proteins, such as protein kinase A, then induce the intracellular effects associated with the various hormones by catalysing the transfer of the terminal phosphate of ATP to target proteins (Fig. 1) (29, 35, 198).

Another important system involves the formation of active molecules from the membrane phospholipids, following the binding of the hormone to a membrane receptor. Activation occurs by the nucleotide regulatory complex Gqα. Although the overall process is analogous to that of adenylate cyclase, a different membrane bound enzyme, phospholipase C, is the catalytic unit. This enzyme acts on a membrane phospholipid, phosphatidylinositol 4,5-diphosphate (PIP2), to produce inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 influences intracellular calcium levels and the other product formed from PIP2, DAG, activates the membrane-bound enzyme protein kinase C

(PKC). PKC phosphorylates intracellular proteins which can then act on a variety of metabolic pathways within the cell, both in the cytoplasm and in the nucleus, to produce the actions associated with the initiator hormone (Fig. 1) (29, 35, 198).

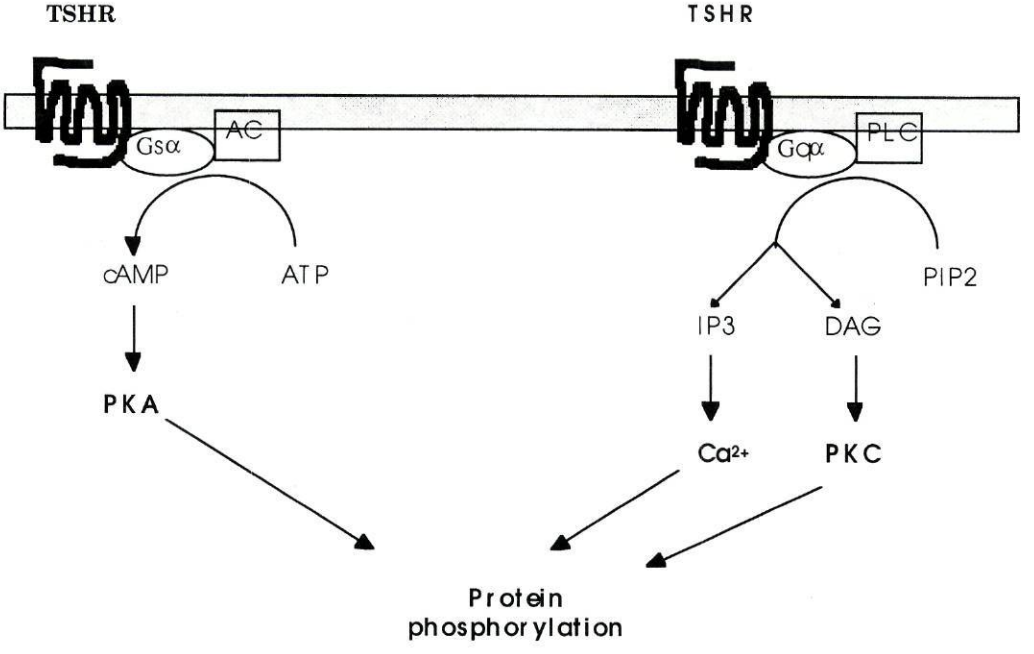


Figure 1- Transducing signal pathways activated by TSHR

Tyrosine kinases receptors (TKR)

Some membrane receptors have inherent tyrosine kinase activity. Consequently, when the appropriate ligand binds to this type of receptor, phosphorylation of specific cytoplasmatic proteins occurs. One effect invariably associated with activation of tyrosine kinase receptors is dimerization and auto-phosphorylation in the tyrosines of the receptor itself which may have positive or negative regulatory effects on the inherent tyrosine kinase activity of the receptor (29). Several TKR are important regulators of thyroid growth and function namely EGFR and IGF-IR.

TKR comprise an extracellular domain containing a ligand-binding site, a single hydrophobic transmembrane domain and a cytosolic domain that includes a region with tyrosine kinase activity. Two different classes of proteins are associated with the cytosolic domain of activated TKR: 1) adapter proteins (e.g. GRB2) that couple the activated receptors to other signalling molecules but have no intrinsic signalling properties; 2) enzymes involved in signalling pathways (e.g. GAP, phospholipase C). Following activation of the TKR a complex containing the activated TKR, the adapter protein GRB2, and a guanine-exchange factor named Sos is formed on the cytosolic face of the plasma membrane. Formation of this complex depends on the dual binding ability of GRB2 through their SH2 and SH3 domains. Receptor activation leads to relocalization of Sos from the cytoplasm to the membrane, bringing Sos near to its substrate, membrane bound ras protein.

Ras is a small guanine nucleotide binding protein (Gsα -300aa; ras-170aa) whose three dimensional structure is similar to that of the part of Gsα that binds GTP. Activation of ras, which is triggered by binding of many growth factors to TKR, occurs in two steps: dissociation of the bound GDP followed by binding of GTP. The first reaction is accelerated by a guanine-exchange factor, like Sos, which binds to the ras-GDP complex, causing dissociation of the bound GDP. SOS thus functions as an activator that promotes the formation of the active ras-GTP complex (29, 198).

A conserved cascade of proteins kinases operates in sequential fashion downstream from activated ras: a) Activated ras binds to the N-terminal domain of raf, a serine-threonine kinase, localizing this cytosolic protein to the plasma membrane; b) the C-terminal end of raf binds to and phosphorylates MEK, a dual specificity protein kinase that phosphorylates both tyrosine and serine residues; c) MEK phosphorylates and activates MAP kinase, another serine/threonine kinase; d) MAP kinase phosphorylates many different proteins including transcription factors that regulate the expression of major cell-cycle and differentiation specific proteins (Fig. 2) (29, 198).

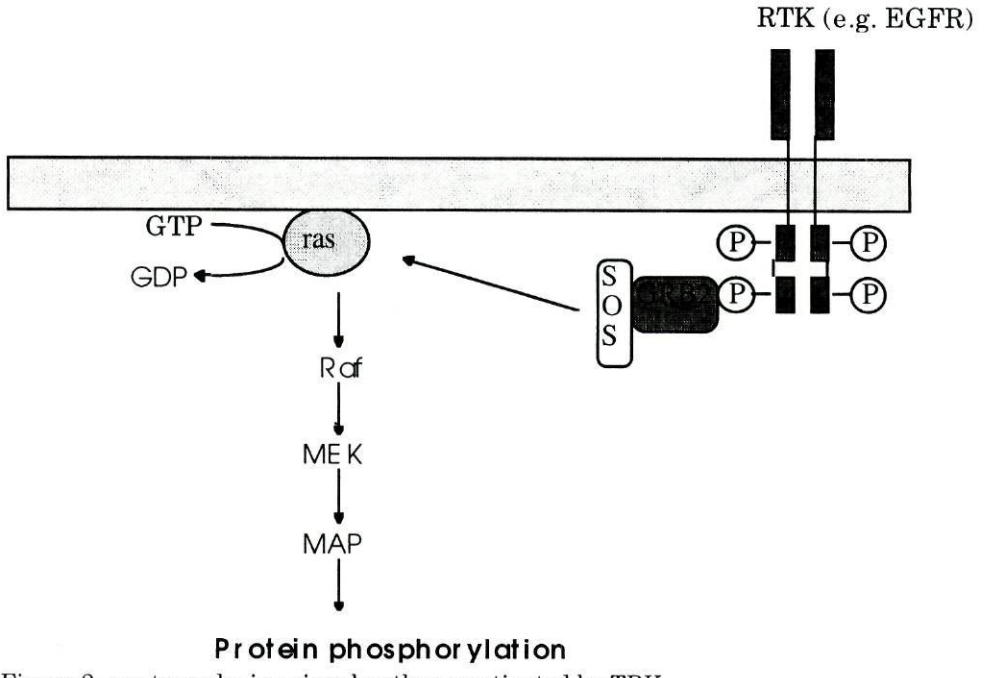


Figure 2- ras transducing signal pathway activated by TRK.

The G-protein linked receptors and the tyrosine kinase receptors can activate different second messengers apart from those in the main pathways previously described for TSHR and EGFR. The relevance of the alternative second messengers in thyroid cell signalling and transduction remains to be elucidated

Regulators of thyroid growth and function

Thyroid gland remains at the same size throughout adult life. The fetal thyroid weighs about 0.2 g at 20-25 weeks and 20-30 g in the adult; assuming a grossly similar tissue composition this growth requires at least 6-7 cell divisions. It was demonstrated by Dumont and collaborators that human thyroid cells divide about five times in adult life with cell division and cell death compensating each other (43). It is not clear if only a sub-population of stem cells has retained this capacity or if all thyrocytes can proliferate under the appropriate stimulus. It is accepted that adult thyrocytes submitted to chronic stimulation are able to grow by hypertrophy and proliferation; however this capacity is limited since, at least in animal models, after 3 months, growth stops in response to TSH stimulation. Similar observations were made in cell cultures (10, 43, 164).

Studies in thyroid cell cultures further showed that growth arrest was not accompanied by morphologic features of senescence, at variance with what happens with fibroblasts (10, 225). Based on these findings Wynford-Thomas, 1997, suggested that thyroid growth arrest might represent a differentiation switch rather than a "timed" senescence mechanism. Recently it was shown that thyrocytes can be rescued from growth arrest by HPVE7 which abrogates Rb action, implying that Rb might be crucial for thyrocyte growth arrest. In the same model the proliferation of thyrocytes observed after Rb abrogation is followed by a second growth arrest which is Rb independent. Rarely, this second growth arrest is overcome and is consistently associated with an epithelial-mesenchymal phenotype switch (225).

Besides promoting the proliferation of thyrocytes in culture from several species including man (6, 43, 212), TSH plays a major role inducing thyroid function, namely controlling hormone synthesis and release. Differentiation, as evaluated by iodide transport, thyroid peroxidase activity and thyroglobulin mRNA content or nuclear transcription is induced by TSH. TSH thus controls thyroid growth *in vivo* while maintaining the expression of differentiation (review by Dumont et al., 1991).

Some growth factors have been shown to be mitogenic or co-mitogenic (permissive) for normal thyrocytes *in vitro* namely EGF, IGF-IR and insulin (43, 67, 181).

EGF induces proliferation in human thyrocytes, but at variance with TSH, the action of EGF is accompanied by a general and reversible loss of function assessed by iodide transport and/or thyroglobulin content (6, 144). EGFR are localised in the basolateral membrane of follicular cells together with TSHR and cell-adhesion molecules (and probably other receptor molecules) (144).

IGF-I is a permissive factor in thyroid growth. Having little effect alone in thyroid proliferation IGF-I acts in a synergistic way with TSH that does not affect differentiation (6, 191, 210, 211). It was demonstrated that thyroid follicular cells express IGF-I; this expression is usually restricted to small, "active" follicles (126, 188). Thyroid follicular cells express also low levels of IGF-IR (126, 188). IGF-I secretion is stimulated by TSH thus predicting that IGF-I plays a key physiological role in synergising with the TSH stimulus to promote thyrocyte growth involving the induction of an IGF-I autocrine circuit (30, 192). IGF-I leads to MAPK cascade activation using transducing molecules different from other tyrosine kinases. The phosphorylation of IGF-IR leads to ras activation *via* the cytosolic protein IRS-1, and activation of several proteins, namely GRB2-Sos (24, 119).

It has been proposed a mitogenic effect for insulin in cell cultures. However, it is not clear if this effect is exerted by its own receptors or through IGF-IR, since the expression of insulin receptors in thyroid follicular cells is low or undetectable and insulin has also affinity for IGF-IR (192).

TGF β is normally expressed and secreted by thyroid follicular cells and acts as a potent inhibitor of thyroid growth. Three distinct TGF β cell surface receptor types (TGF βRI, II and III) have been cloned and characterised. Type I and II are transmembrane

serine/threonine kinases, whereas type III receptor is a transmembrane proteoglycans devoid of intrinsic signalling ability which may facilitate binding of TGF β to type II receptor. The most commonly held model for receptor activation proposes that type I and type II receptors form an heteromeric complex essential for signal transdution. TGFβ binds a type II receptor which phosphorylates and activates a type I receptor that then initiates downstream signalling. The recently described TGFβ signal transducing proteins SMAD 2, 3, 4 and 5 are phosphorylated and form heteromeric complex accumulating in the nucleus where they can regulate transcription (123, 217).

Three thyroid specific transcription factors have been identified; TTF-1, TTF-2 and Pax-8 (25, 109, 231). All of them bind and activate the promoter region of thyroid-specific genes, thyroglobulin and TPO, regulating the expression of these genes (100). TSH, IGF-I and insulin increase the transcription and/or DNA binding of the thyroid transcription factors (147), whereas antisense oligonucleotides to TTF-1 and Pax-8 cause a significant reduction in the proliferation induced in FRTL-5 (thyroid cell line) by TSH and IGF-I (158). These data suggest that thyroid specific transcription factors are important in thyroid function as well in thyroid growth.

Growth control and thyroid neoplasia

The role played by signals and cascades controlling thyroid cell proliferation and differentiation is an important area of research in thyroid pathology in general, and thyroid oncology in particular. In a few cases the origin of thyroid tumours can be explained straightforwardly within the frame presented in the above sections.

The best examples concern the TSH pathways *via* the G-protein. There is evidence pointing to a role of TSH in the growth and the behaviour of (some) thyroid tumours, particularly through its mitogenic activity over thyroid cells (200). Clinically, the treatment with thyroxin in order to suppress TSH has been advanced to decrease the risk of thyroid cancer recurrence and to improve patients survival but these results remain disputable (131, 155). TSH stimulates growth and invasion of some

differentiated thyroid cancer cell lines (42, 43). Recently, it was demonstrated the growth stimulatory effect of TSH in thyroid tumours arising in ret/PTC1 transgenic mice (164).

Somatic mutations of the TSHR gene were found in about 50% of hyperfunctioning adenomas (65, 161). Mutations of the transmembrane domain, encoded by exon 10, were reported in a subset of thyroid carcinomas characterized by a high basal level of adenylate cyclase activity and a poor response to TSH (160, 162). These mutations lead to a constitutive activation of TSHR and consequently of the whole cAMP cascade, thus being a stimulus for a continuous growth of the follicular cells (160, 185).

Other element involved in the signal transdution of TSH, Gsα protein, has also been found mutated, at first in autonomously functioning nodules (125, 146) and, afterwards, in thyroid carcinomas (69, 185, 186). It was suggested that thyroid carcinomas with mutations of Gsα proteins belong to the characteristic sub-group of tumours with high levels of basal cAMP (185, 186) and to maintained stimulation due to the oncogenic mutations (185).

Low or undetectable level of TPO immunoreactivity was found in PTC (32, 189, 197) in parallel with low or undetectable TPO mRNA levels, suggesting that suppression of TPO mRNA transcription causes low TPO activity in PTC (189).

The pathways of tyrosine-kinase receptors have been often the target of study in thyroid carcinogenesis particularly regarding those growth factor/growth factor receptors implicated in growth and differentiation of thyroid follicular cells (42, 52, 61).

Members of the EGFR family are frequently found deregulated in thyroid carcinomas and it was advanced the prognostic value of EGFR (over)expression in PTC (3, 134). EGFR has several agonists, at least EGF and TGFα, have been found in normal thyroid, and TGFα was found overexpressed in thyroid carcinomas, and, in particular in PTC

(78, 114). A putative autocrine loop was suggested in PTC leading to an increased expression of the growth stimulatory pathway driven by EGFR (1, 2, 78). However no abnormalities in the EGFR gene and in EGFR ligands were detected; in other words, no structural abnormalities which could be related to the abnormal expression of EGF/TGFα/ EGFR were observed (2, 78, 118)

Another member of EGFR family that has been associated to thyroid neoplasia, particularly to PTC, is c-erb B2 . This receptor still is an orphan receptor. It was advanced that neu differentiation factors could be ligands of c-erb B2 (70) but it was proposed thereafter that c-erb B2 activation results from dimerization with EGFR, erbB3 or erbB4, having all these receptors binding capacity to neu differentiation factors with different levels of affinity (87). C-erb B2 can, at least from a theoretic standpoint, mediate EGFR activation, but it is not known if c-erb B2 has any effect in normal thyroid growth and/or differentiation. Though Lemoine et al did not find c-erb B2 expression or gene alterations in thyroid lesions (118), it has been reported afterwards the specific overexpression of this protein in PTC (77). Cytoplasmic expression of c-erb B2 has been recently associated with PTC of young patients and better survival (3). (see Discussion Section)

IGF-I is secreted by human thyroid cells and this secretion is stimulated by TSH (192). It was verified that transformation of thyroid cells with mutant H-ras leads to IGF-I secretion (31). Conditioned medium from thyroid adenoma cells in culture confers IGF-I independence to normal thyroid cells (210, 211) due to autocrine production of IGF-I by the adenoma cells, thus suggesting that autocrine production of IGF-I may be an initial step in thyroid oncogenesis. In contrast, it was found in the majority of PTC a weak to moderate expression of IGF-I and IGF-IR (188). The same does not hold true regarding a subset of PTC presenting an abundant lymphoplasmacytic infiltrate in which a uniformly strong positivity for IGF-IR was detected (188). Interestingly, the lymphoid cells infiltrating these tumours were strongly positive for IGF-I mRNA (188). These

findings suggest that in this particular subset of PTC a symbiotic relationship between the tumour cells and the lymphoid cells may exist (188).

As stated above ras is an important element in the transduction of signals from activated tyrosine kinase receptors. The putative role of ras in papillary carcinogenesis is controversial, since the different series on record report very different frequencies of ras activation (4, 73, 102, 115, 116, 135, 137, 138, 148, 175, 187, 219, 228) and some authors did not even find any activation of ras at all in PTC (127). There is a general agreement on the much lower frequency of ras activation in papillary carcinomas than in follicular carcinomas (115, 116, 219). One possible explanation advanced by some authors to explain this difference lies in the fact that most papillary carcinomas arise as *de novo* lesions whereas follicular carcinomas usually arise from preexisting follicular adenomas (115). The higher rate of ras mutations in follicular carcinomas may thus reflect their origin from adenomas, in which ras activation is relatively frequent and presumably plays a key etiopathogenic role (115, 117).

It was also advanced that the difference in ras oncogene activation between follicular and papillary carcinoma might be related to the different metastatic potential of follicular carcinomas which disseminate by the blood stream at variance with PTC which invade locally and *via* lymphatics (127, 219). In one study ras immunohistochemical expression was correlated with the degree of aggressiveness of PTC (4), in this study there was, however, no correlation with the presence of distant metastases.

Ret is a transmembrane tyrosine kinase protein signalling for neurotrophic factors — Glial-cell-line-derived neurotrophic factor (GDNF) and neurturin (NTN) — through a multicomponent receptor (104, 194). This receptor is composed of a shared transmembrane protein tyrosine kinase (ret protein) and a ligand-specific GPI-linked protein (104). Ret proto-oncogene is expressed during development in excretory and in the peripheral and central nervous systems; in thyroid only the C-cells express ret (49).

Ret gene, located in chromosome 10q11.2, has been implicated in hereditary and sporadic cancer and its activation can result from mutation or rearrangement (40, 88, 107). Point mutations in the different regions of the gene are associated to different cancer syndromes (199). A missense mutation in the cysteine rich domain is involved in the majority of the MEN2A tumours. This cystein rich region is also frequently involved in (isolated) familial medullary thyroid carcinoma (FMTC). Almost all the cases of MEN2B show alteration in the methionine in codon 918. Mutations in ret oncogene have also been found in Hirshprung disease, where they cause loss or truncation of ret protein. In contrast to Hirshprung disease the mutations occurring in MEN 2A, MEN 2B and FMTC lead to oncogenic activation of ret by change in cytoplasmic substrate (in MEN 2B) or increased oligomerization (in MEN 2A and FMTC) (For a review see Komminoth, 1997).

Ret rearrangement is found, overall, in about 30% of PTC but the frequency of the rearrangement varies widely among the series (12, 13, 64, 71, 95, 105, 139, 167, 168, 169, 184, 206, 214, 215). Ret rearrangement has never been described to the best of our knowledge outside PTC, thus representing a unique example of organ and tumour specificity. Although different types of rearrangement have been described to date they share some common features: the breakpoint in ret gene is usually in the intron 11, the genes involved in the rearrangement are normally expressed in the thyroid and the translocated region has dimerization domains (89, 110, 193).

The rearrangement leads to the expression and dimerization in the cytoplasm of ret tyrosine kinase domain. The dimerization results in autophosphorylation of the kinase domains in the absence of the ligand for ret (89, 110). It is not known if the different forms of rearrangements have different oncogenic effects (107).

Trk a tyrosine kinase receptor for nerve growth factor has also been found rearranged in PTC by a similar mechanism as ret. In the majority of the cases intrachromosomal rearrangements involving trk gene and tropomyosin gene both located in chromosome 1

lead to the formation of trk derived oncogenes (13, 133). Although few data is available about trk rearrangements in PTC, its frequency seems to be much lower than those ones involving ret oncogene. No further information is available in the phenotypical characterization of these tumours, except for the higher frequency of trk rearrangements in PTC from younger patients (12).

A role of tumour suppressor gene has been attributed to TGFβR in epithelial models. In thyroid TGFβ acts as a potent inhibitor of cell growth and it is therefore likely that genetic alterations that induce modifications in the expression in either TGFβ or TGFβR may result in cellular growth advantage and consequently serve as a step in thyroid carcinogenesis (123). Loss of TGFβ responsiveness was observed in advanced stages of thyroid tumours (221) although a retained sensitivity to the growth inhibitory effect of TGFβ was observed in papillary thyroid carcinomas *in vitro* (8). Furthermore, in a model of ras-transfected thyroid cell line, loss of responsiveness to TGF β was associated with tumourigenicity in nude mice (8, 9). A reduced amount of mRNA and protein expression of TGFβR was found in a series of thyroid carcinomas when compared with their relative normal counterparts (112). The greatest reduction in TGFβR expression was found in an undifferentiated carcinoma (112). No alterations in the gene structure were detected in any of the cases, thus suggesting that abnormalities in the transcriptional regulation of TGFβR gene might be involved in the escape from TGFβ growth control (112).

P53 has been extensively studied in thyroid tumours (37, 38, 39, 50, 51, 84, 86, 91, 130, 136, 218, 223, 229). Being found overexpressed/mutated in the majority of the undifferentiated carcinomas, a key role in the transition to the undifferentiated type was attributed to p53 mutation (39, 90, 91), whereas only few studies reported the existence of alterations of this protein in differentiated tumours (55, 165). *In vitro* reintroduction of wild-type p53 in thyroid carcinoma cell lines harbouring an inactivated p53 leads to the expression of differentiated features, namely the reexpression of thyroid peroxidase (51). These findings point to a role of p53 in the

differentiation of follicular cells. This effect seems to be exerted *via* the activation of the thyroid specific transcription factor Pax-8 (51)

The tumour supressor gene for retinoblastoma susceptibility (RB) was also found frequently anomalous in a series of PTC (52). This finding fits with the notion that RB gene plays a major regulator role in follicular cell proliferation since abrogation of RB function by HPVE7 leads to hyperplasia and tumour development in the thyroid (225). Despite this, in immunohistochemical studies no abnormal expression of RB protein was detected (86).

Several proteins involved in the regulation of cell cycle such as inhibitors of cyclin dependent kinases were analysed in thyroid lesions at the gene and/or protein level (20, 92, 124). Reduced expression of p27, a protein that regulates cell cycle progression from G1 into S phase by binding to and inhibiting the cyclin E/Cdk2 complex, was found in thyroid carcinomas when compared to normal thyroid and follicular adenomas (124). Concerning p16, no gene or expression alterations were found in a series of primary thyroid tumours, whereas different types of inactivation (deletions, point mutations and methylation of CpG islands in the exon 1) were described in thyroid cell lines of the papillary and follicular histotype (20, 94, 98). These findings suggest that, despite not being directly involved in the process of tumourigenesis in thyroid, p16 probably gives selective growth advantage in tissue culture (98). Shi et al., 1996, found evidence of deletion of WAF1/p21 transcript at the mRNA level in a minority of PTC, due probably to abnormal RNA splicing since no gene deletions or rearrangements were found at the genomic level (174). In contrast to normal thyroid, consistent immunohistochemical expression of WAF/p21 was found in thyroid tumours, including PTC, but no correlation with clinical or prognostic parameters could be established (92)

A large number of molecules has been studied in thyroid cells in both normal and pathologic situations. It would be impossible to review and refer to all the data on record on thyroid oncogenesis in this short review. We are aware that important or

putatively important factors in several domains were not addressed so far. However we decided to refer only to those ones that appear to be more consistently involved in thyroid papillary oncogenesis. Some of the factors either omitted or only briefly referred until now will be referred in the Discussion Section.

AIMS

From the model of development of thyroid tumours proposed by Wynford-Thomas (224) two distinct routes involving different initiating genetic events would lead to the two major types of differentiated thyroid carcinoma: ras mutation would act as the initial event for the follicular carcinogenesis and ret rearrangement as the initial event for papillary carcinogenesis (224). It is generally accepted that p53 mutations are a key event in the establishment of undifferentiated carcinomas from pre-existing differentiated types (39, 91, 224). We have focused our work on PTC, not only because we were particularly interested in the study of this type of thyroid carcinoma but also because it would be impossible to follow the natural history of other types of thyroid carcinoma; whenever necessary, however, we have also analysed, for comparative purposes, other thyroid lesions.

Furthermore, we have concentrated our study, for practical reasons, only on some of the factors putatively linked to the etiopathogenesis and development of PTC. The criteria used in the selection of the aforementioned factors stem from our interest in addressing the issue of the dissociation between growth and invasiveness in PTC rather than from any quantitative evaluation of the relative importance of the molecular biology features of PTC oncogenesis. (In other words, we do not even know if the features we have selected for study are the most important in terms of the pathobiology of PTC).

The capacity of tiny micropapillary carcinomas to give rise to metastases in regional lymph nodes is the clearest manifestation of the dissociation between growth and invasiveness in PTC. The consistently low proliferative rate of PTC regardless of the metastatic status reinforces such dissociation.

PTC frequently occurs in young people who frequently present lymph node involvement and, less often, though not rarely, lung metastases. Even in this setting PTC are potentially curable, partly because the neoplastic cells retain usually their capacity on trapping radioactive iodine, almost like normal cells, and partly because the metastatic

ability of PTC is not accompanied in most cases by other features of clinical aggressiveness, thus showing, once more, the peculiarities of PTC behaviour.

Genetic alterations in the main regulators of thyroid growth (TSH, EGF and IGF-I) have been rarely, if ever, found in PTC. Mutations in the genes of the TSH receptor/Gsα pathway have only been reported in an extremely rare subset of PTC, the so-called hyperfunctioning PTC (146, 160). At variance with this, PTC exhibits major changes in tyrosine kinase receptors with two main types of alterations: gene rearrangement with *de novo* expression in the cases of ret and trk proto-oncogene (13) and overexpression without apparent structural genetic alterations of several growth factor/growth factor receptors such as EGFR and c-met/HGFR (3, 36). In an oversimplified way we could summarize the aforementioned data stressing that, apart from ret and trk, very few genetic alterations have been ascribed to PTC; in contrast to this, numerous alterations in the expression of growth factors/growth factor receptors are frequently found in PTC. Taking these features into account, together with the expertise of our Institute and our own goals, we decided to build up the present study addressing the following four main questions:

1. Is proliferation/genomic stability deregulated in PTC?

The quantification of thyroid proliferation is crucial to determine to what extent growth is deregulated in thyroid pathology. The use of immunohistochemical markers of cell proliferation appeared to be a reasonable method to deal with clinical samples. For this purpose we evaluated the proliferation indexes of thyroid lesions in general, and PTC in particular, using two proliferation-related immunohistochemical markers, PCNA and MIB1.

An attempt to address the apoptosis "arm" of PTC development was also undertaken through the evaluation of the expression of bcl2 protein .

Deregulated growth can result from alterations in genes that control genomic stability, namely in genes involved in cell cycle control or in genes involved in DNA stability. In an attempt to evaluate the genomic stability status in thyroid tumours, p53 which is known to be involved in cell cycle control as well as a tumour suppressor gene in neoplasia, was studied. Genomic instability resulting from alterations of genes involved in DNA mismatch repair has been recently reported in hereditary and sporadic tumours. Alterations at microsatellite loci have been described as a phenotypic marker of these tumours usually designated as MI tumours (mutator phenotype). This type of genomic stability could also be deregulated in thyroid papillary carcinomas. Having in mind that p53 and MI are thought to represent two distinct mechanisms in carcinogenesis — the suppressor and the mutator pathway — we studied both of them in an attempt to find if any of these two mechanisms of carcinogenesis (or both) are operative in thyroid tumours, as described in tumours of other organs.

2. Are growth factor/growth factor receptors specifically deregulated in PTC (or in a subset of PTC) at gene/expression level?

In an endocrine organ where cell-cell and tissue-organism communication has a key importance, alterations of growth factor/growth factor receptors are known to play a major role in the neoplastic development and inherent growth and differentiation deregulation. In this field we decided to select c-erb b2 and c-met/HGFR as main targets for study both at the expression and at gene level.

3. Is the particular invasive/metastatic pattern of PTC related to alterations in the epithelial-adhesion molecule E-cadherin?

It seems logical to assume that in thyroid carcinogenesis, like in the neoplastic development of other organs, cell-adhesion systems may play a role. Such systems are likely to be particularly important in a neoplasia characterised by local invasiveness and a high frequency of lymph node involvement at diagnosis like PTC. As a key molecule of cell adhesion in epithelial systems E-cadherin was studied both at the

expression and gene level. Vimentin expression, an intermediate filament putatively related to the metastatic ability was also evaluated.

4. What does ret activation mean from a clinical and a pathobiological standpoint?

The scarcity of data relating the biological impact of the activation of ret oncogene, the sole genetic alteration which can be attributed specifically to PTC, with the clinical behaviour of the tumours, pushed us to investigate such relationship. For that, selected features of PTC with and without ret rearrangement were compared from a clinical and a pathobiological standpoint.

MATERIAL AND METHODS

The methods used in the different studies are described in detail in Papers I to VI. Briefly, the following methods were used:

Immunohistochemistry - Used in the study of the expression of c-erb B2, p53, E-cadherin, MIB-1, PCNA, bcl2, c-met/HGFR , vimentin, cytokeratins, thyroglobulin (Papers I - IV and VI). The immunohistochemistry study of uPAR was performed following the same methodology described in Papers I to IV and VI, using a polyclonal antibody (Kind offer from Doctor Ebbe Rønne).

Southern-blot - Used in the study of ret rearrangement, and of c-met/HGFR and c-erb B2 genes (Papers II and VI).

Western-blot - Used in the characterisation of c-erb B2 protein (Paper II).

PCR (polymerase chain reaction) - Used in the analysis of dinucleotide microsatellites (search of MI) and E-cadherin gene (Papers IV and V).

SSCP (single strand conformation polymorphism) analysis - Used in the screening of mutations of the E-cadherin gene (Paper IV).

CDGE (constant denaturating gel electrophoresis) - Used in the screening of mutations of the hMSH2 gene (the analysis was performed at Radium Hospital by the co-authors of paper) (Paper V).

Sequencing - Used in the DNA sequencing of the E-cadherin gene, using both automated and manual techniques (Paper IV).

Methodological considerations will be included in the discussion when appropriate.

The biological material included in the different studies is described in Papers I-VI; due to the diversity of the lesions used in each of these studies we will omit now their description.

RESULTS

The results are presented in detail in Papers I to VI. A summary will be presented here stressing the most interesting findings in relation to the issues raised in the Aims Section.

Proliferation - The proliferative index of PTC is low and similar to those of benign lesions and follicular carcinomas evaluated through PCNA immunoreactivity (Table 1) (Paper I). This finding was confirmed by the study of Ki-67 using MIB-1 antibody (Fig. 3A). The mean value of MIB-1 in PTC was 1.45% (min-0.2; max-4.5; SE-0.7) (Paper VI).

Table 1 - Summary of the data on the immunoreactivity for PCNA in thyroid lesions

Diagnoses (n)	Mean (SE) %	p value*
Benign lesion (13)	3.6 (1.9)	NS**
Papillary carcinoma (12)	4.4 (1.5)	NS**
Follicular carcinoma (9)	2.9 (1.2)	NS**
Poorly differentiated carcinoma (30)	11.5 (2.2)	< 0.05***
Undifferentiated carcinoma (12)	38.7 (3.9)	< 0.01***

* Calculated using ANOVA and Student's t test.
** No significant differences between benign lesions and both types of well differentiated carcinomas.
***In comparison to benign lesions and well differentiated carcinomas. For details see Paper I

Proliferative index obtained by PCNA and MIB-1 were compared in a series of 10 cases of follicular adenoma and adjacent thyroid (r=0.67)(unpublished results)(Table 2).

Table 2- PCNA and MIB-1 percentage of immunoreactive cells in 10 thyroid lesions and adjacent thyroid

	MIB-1(%)		PCNA (%)	
CASE	Normal thyroid	Adenoma	Normal thyroid	Adenoma
1	0	0.6	1.5	4.6
2	0	1.5	0.3	2
3	0.1	1	2.1	5.2
4	0.1	0.5	1.4	2.8
5	0.1	0.5	0.5	5.4
6	0.2	0.4	2	2.4
7	0	0.5	0.7	2.3
8	0.6	1	1.4	6.8
9	0.2	0.3	0.9	1.7
10	0.9	0.8	1	1.2

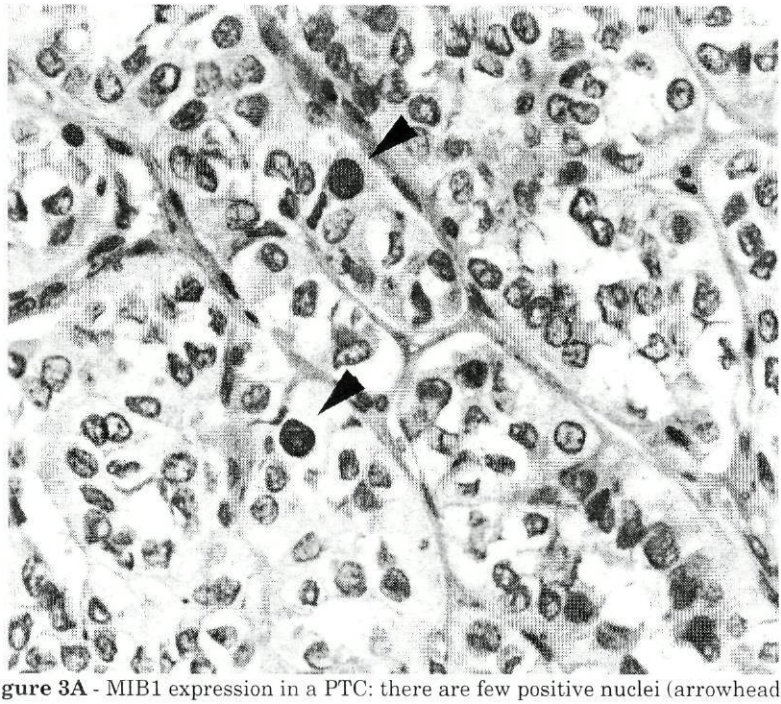


Figure 3A - MIB1 expression in a PTC: there are few positive nuclei (arrowheads).

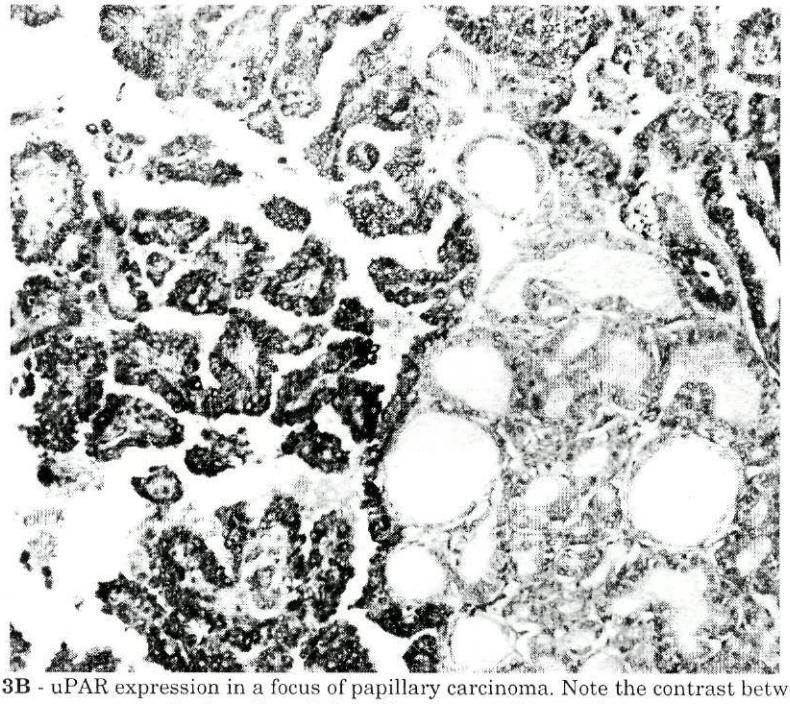


Figure 3B - uPAR expression in a focus of papillary carcinoma. Note the contrast between the neoplastic cells and the adjacent normal parenchyma.

Bcl 2 - Immunoreactivity for bcl-2 was observed in most PTC as well as in the normal adjacent thyroid (Paper VI). The immunoreactivity was cytoplasmic and usually less intense in the carcinomas than in the normal thyroid.

P53 - No nuclear immunoreactivity for p53 was found in any PTC at variance with its finding in a few widely invasive follicular carcinomas and poorly differentiated carcinomas and in many undifferentiated carcinomas (Table 3) (Paper III)

Table 3 - Summary of the data on the immunoreactivity for p53 in thyroid lesions

Diagnoses (n)	Staining Score*			
	Neg n(%)	< 10% n(%)	≥10 <50% n(%)	≥50% n(%)
Nodular goitre and adenoma (14)	14 (100)	0(0)	0(0)	0(0)
Papillary carcinoma (12)	12 (100)	0(0)	0(0)	0(0)
Follicular carcinoma (10)	8 (80)	2(20)	0(0)	0(0)
Poorly differentiated. carcinoma (31)	26 (84)	4(13)	1(3)	0(0)
Undifferentiated carcinoma (12)	2 (16)	2(16)	6(50)	2(16)

*Percentage of immunoreactive cells. For details see Paper III

Microsatellite instability (MI) - The prevalence of MI is low in PTC and apparently does not differ from those observed in benign lesions and other types of carcinoma (Table 4) (Paper IV). No mutations were found in the hMSH2 repair gene in the cases which were positive for MI (Paper IV).

Table 4 - Frequency of microsatellite instability in thyroid lesions

Diagnoses (n)	MI≥1 n(%)	MI≥3 n(%)
Nodular goitre (13)	2 (15.4)	1 (7.6)
Follicular adenoma (15)	2 (13.3)	1 (6.6)
Papillary carcinoma (12)	2 (16.7)	1 (8.3)
Follicular carcinoma (4)	1 (25.0)	0 (0)
Poorly differentiated carcinoma (2)	0 (0)	0 (0)

For details see Paper IV

c-erb B2 - No gene alterations (amplification or rearrangement) were found by Southern-blot. Immunoreactivity for c-erb B2 in PTC, as well in other thyroid lesions and in the normal thyroid, was always cytoplasmic. No significant differences were found in the intensity of the staining and percentage of immunoreactive cells between PTC and other benign and malignant thyroid lesions (Table 5) (Paper II). By Western-blot the c-erb B2 antibodies identify a protein with lower molecular weight than that reported for the mature form of c-erb B2.

Table 5 - Summary of the data on the immunoreactivity for c-erb B2 in thyroid lesions

Diagnoses (n)	Staining Score			
	Neg n(%)	< 10% n(%)	≥10 <50% n(%)	≥50% n(%)
Nodular goitre (4)	0 (0)	0 (0)	2 (50)	2 (50)
Adenoma (6)	0 (0)	4 (66)	1 (17)	1 (17)
Follicular carcinoma (4)	0 (0)	2 (50)	1 (25)	1 (25)
Papillary carcinoma (10)	1 (10)	4 (40)	4 (40)	1 (10)
Poorly differentiated carcinoma (6)	2 (33)	1 (17)	2 (33)	1 (17)
Undifferentiated carcinoma (1)	0 (0)	1 (100)	0 (0)	0 (0)
Medullary carcinoma (3)	2 (66)	0 (0)	1 (33)	0 (0)
Normal thyroid (25)	1 (4)	12 (48)	7 (28)	5 (20)

* Percentage of immunoreactive cells. For details see Paper II

c-met/HGFR - No gene alterations (amplification or rearrangement) were found by Southern-blot in any PTC (Fig. 4). The immunoreactivity for c-met/HGFR in PTC was cytoplasmic and the majority of the PTC were positive for c-met/HGFR protein (Paper VI) in contrast to the absence of immunostaining in the adjacent normal thyroid. A positive correlation was found between the expression of c-met/HGFR and vimentin in PTC ($r = 0.6$; $p = 0.002$)

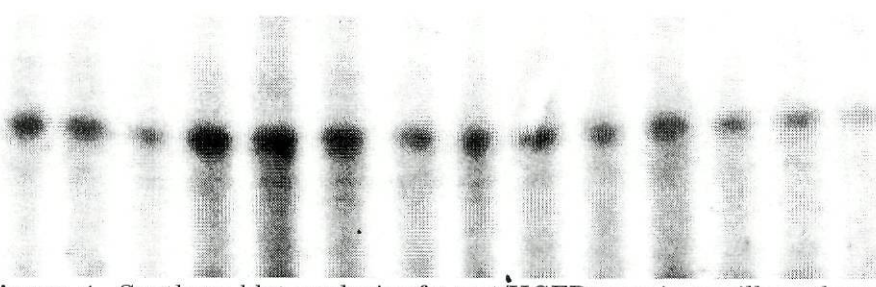


Figure 4 - Southern blot analysis of c-met/HGFR gene in papillary thyroid carcinomas. DNA samples were digested with EcoRI and hybridized to c-met/HGFR probe.

E-cadherin - Mutation of E-cadherin gene was found in 1 out of 14 PTC (7%). The mutated case presented a missense mutation at exon 12 of the E-cadherin gene and was classified histologically as a diffuse sclerosing variant of PTC. Immunoreactivity for E-cadherin was detected in normal thyroid as well as in the majority of all the types of lesions (Table 6) (Paper V). PTC presents frequently low and heterogeneous immunoreactivity. The immunoreactivity of the mutated PTC did not apparently differ from that of the remaining PTC (Paper V)

Table 6 - Summary of the data on the immunoreactivity for E-cadherin in thyroid lesions

	Membrane*					Cytoplasm*				
Diagnoses (n)	**0**	**0/+**	**+**	**++**	**+++**	**0**	**0/+**	**+**	**++**	**+++**
Follicular adenoma (2)				2		1			1	
Papillary carcinoma (17)		3	9	3	2	3	6	3	5	
Follicular carcinoma (8)				3	5	3	1	3	1	
Poorly differentiated carcinoma (3)	2		1				2	1		
Nodal metastasis from PTC (7)		3	4			1	5	1		

* Intensity of the staining. For details see Paper V

uPAR- All the PTC presented cytoplasmic uPAR overexpression in contrast to the adjacent thyroid (Fig. 3B) (Table 7). The immunohistochemical score was significantly higher ($p = 0.02$) in cases presenting venous invasion than in the remaining PTC (Table 8). Tumours greater than 3 cm showed a tendency for strong immunoreactivity ($p = 0.08$) (Table 8).

Table 7 - Summary of the data on the immunoreactivity for expression of uPAR in a series of 31 PTC.

Percentage of immunoreactive cells	<25%	≥25 <50%	≥50 <75%	≥75%
n (%)	0 (0)	8 (26)	8 (26)	15 (48)
Intensity	0/+	+	++	+++
n(%)	6 (19)	9 (29)	13 (42)	3 (10)

Table 8 - Comparison between the uPAR staining score and clinicopathological data in a series of PTC.

	Staining Score* (n)	p value**
Gender		NS
Male	10.0 (5)	
Female	7.9 (26)	
Age		NS
<45y	8.0 (20)	
≥45y	8.7 (11)	
Tumour diameter		NS
<3 cm	7.2 (19)	(p=0.08)
≥3 cm	10.4 (10)	
Tumour proliferative rate		NS
<1.5%	8 .0 (19)	
≥1.5%	8.7 (12)	
Multicentricity		NS
Absent	7.5 (11)	
Present	8.3 (16)	
Extrathyroidal growth		NS
Absent	7.6 (21)	
Present	9.3 (7)	
Vascular invasion		p=0.02
Absent	6.7 (20)	
Present	11.3 (8)	
Lymph node involvement		NS
Absent	7.9 (17)	
Present	8.7 (14)	

*The staining score was determined as described in Paper VI. For details see Paper VI
** Calculated using ANOVA and Student's t test

ret - Rearrangements of ret were found in 24.2% of PTC. The mean age of the patients with tumours displaying ret rearrangement was significantly lower (p=0.005) than that of the patients harbouring cases that did not present rearrangement (Table 9). The proliferative rate of PTC with ret rearrangement was suggestively lower (p=0.09) than that of PTC without ret rearrangement (Scattergram 1) (Paper VI).

No significant differences were found between cases of PTC with and without ret rearrangement in relation to bcl-2 expression (Table 10) (Paper VI).

The comparison of c-erb B2 immunoreactivity in cases of PTC with and without ret rearrangement did not reveal any significant differences (Table 10) (Paper VI).

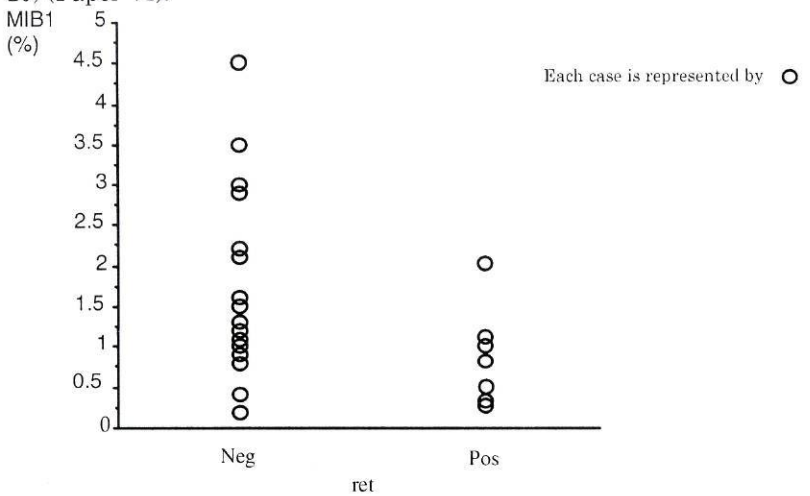
In the comparison of c-met/HGFR immunoreactivity in cases of PTC with and without ret rearrangement no differences were also found (Table 10) (Paper VI).

Cases of PTC with ret rearrangement showed a significantly lower (p=0.007) cytoplasmic immunoreactivity for E-cadherin than cases without rearrangement (Scattergram 2) (Table 10) (Paper VI).

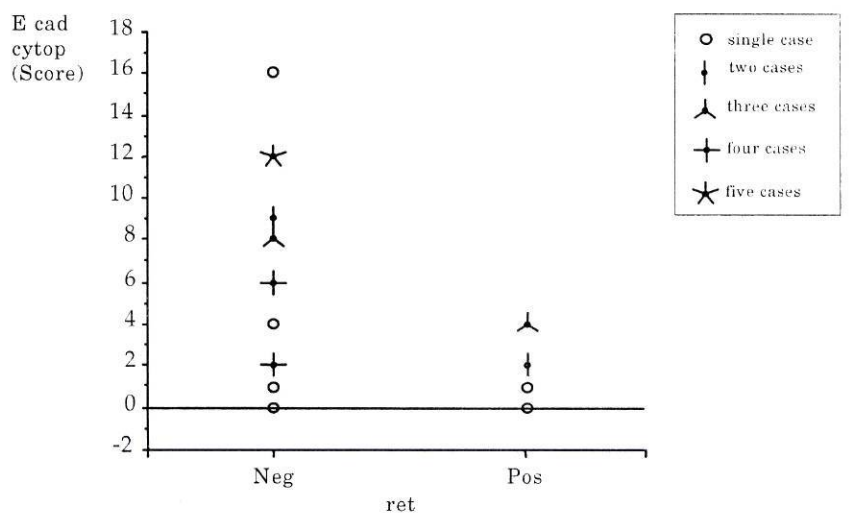
Almost every PTC (81%) displayed immunoreactivity for vimentin in contrast to the adjacent normal thyroid (Paper VI). The expression of vimentin was localised in the basal pole of the cancer cells in the large majority of PTC regardless of the presence or absence of ret rearrangement (Table 10) (Paper VI).

No significant differences were found between cases of PTC with and without rearrangement regarding the percentage of immunoreactive cells, the intensity of staining and the staining score for uPAR (Table 10).

No differences between PTC with and without ret rearrangement were found in relation to the other clinicopathological and immunohistochemical features studied (Tables 9 and 10) (Paper VI).



Scattergram 1 - MIB-1 proliferative rate in cases of PTC with and without ret rearrangement



Scattergram 2 - E-cadherin cytoplasmic immunoreactivity in cases of PTC with and without ret rearrangement.

Table 9 - Comparison of the two groups of PTC with and without ret rearrangement regarding the clinicopathological data

	Rearranged ret n=8	Non-rearranged ret n=25	p value*
Gender (M:F)	1:7	1:3.2	NS
Age (y) (Mean± SE)	28.1 (± 3.1)	44.7 (± 2.9)	p = 0.005
Size (cm)(Mean±SE)	3.3 (± 0.9)	3.3 (± 0.6)	NS
Proliferative rate (%)(Mean±SE)	1.0 (± 0.2)	1.6 (± 0.2)	NS (p=0.1)
Histologic subtype			
Predominantly papillary (n=22)	87.5%	60.0%	
Follicular variant (n=11)	12.5%	40.0%	NS (p = 0.15)
Multicentricity	75.0%	59.1%	NS
Extrathyroidal growth	25.0%	24.0%	NS
Vascular invasion	25.0%	24.0%	NS
Lymphocytic infiltration			
Absent	28.6%	17.4%	
Discrete	71.4%	56.5%	
Moderate/abundant	0	26.1%	NS
Lymph node involvement*	62.5%	36.0%	NS (p=0.19)

* Calculated using ANOVA and Student's t test
For details see Paper VI

Table 10 - Summary of the immunohistochemical scores in PTC with and without ret rearrangement

Immunohistochemical score	Rearranged ret	Non-rearranged ret	p value*
E-cadherin - membrane (SE)	6.0 (2.0)	8. (1.0)	NS
E-cadherin - cytoplasm (SE)	2.7 (0.7)	8.0 (1.1)	0.007
c-erb B2 (SE)	10.3 (1.8)	6.7 (0.8)	NS
c-met/HGFR (SE)	8.8 (2.6)	10.9 (1.2)	NS
bcl-2 (SE)	3.6 (1.9)	6.2 (1.1)	NS
vimentin (SE)	7.3 (1.7)	6.6 (1.1)	NS
uPAR (SE)	8.4(1.2)	8.2(0.9)	NS

* Calculated using ANOVA and Student's t test
For details see Paper VI

DISCUSSION

For the sake of simplicity we have organized the Discussion around the issues raised in the Aims Section puting a special emphasis on those we have some personal experience.

1. Is proliferation/genomic stability deregulated in PTC?

Our results fit with the low **proliferative rate** of differentiated thyroid carcinomas, as it had been previously demonstrated using several methodologies (5, 103, 149, 153, 157, 176, 208, 227). Moreover, we and others (149, 227), at variance with Shimizu et al., 1993, were unable to find significant differences between the proliferative rate of benign lesions and PTC. Our findings thus contrast with those on other tumour models in which a clear cut difference is found whenever one compares benign lesions with their malignant counterparts.

PCNA was the first immunohistochemical marker of proliferation that was found to be operative in paraffin sections. Soon it became evident that it led to an overestimation of the proliferative rate (132). Indeed, as an auxiliary protein of polymerase-δ, PCNA is implied not only in DNA replication but also in DNA repair (85, 132). Another major drawback of PCNA rests in the long half life of the protein leading to its immunohistochemical detection in cells who are already out of cycle (85, 132). The finding of Ki-67, a protein expressed in a cell cycle dependent manner, abridge the overestimation of PCNA, but its use has been for a while restricted to frozen sections. The emergence of anti Ki-67 antibodies working in paraffin sections (**MIB-1**) allowed its detection in sections obtained from paraffin blocks (85). Understandably, we employed PCNA in our first assays and MIB-1 subsequently. The results obtained using these two methods in the evaluation of the proliferative rate were compared in a small group of lesions in which both antibodies were used (Table 2); this comparison yielded similar results with the two antibodies though the proliferative rate evaluated by PCNA was, as expected, usually higher than the proliferative rate evaluated by MIB-1.

We found a complete overlapping of the proliferative indexes of benign lesions (nodular goitres and adenomas) and differentiated carcinomas (papillary and follicular carcinomas). Within these groups, the highest score was found in a nodular goitre thus

contributing to rule out the usefulness of the evaluation of the proliferative activity for the distinction of benign and malignant thyroid lesions. These results were confirmed by a parallel DNA flow cytometric study of frozen material (data not shown) and fit with the reports of several groups on the same issue (5, 103, 149, 153, 176, 227).

High proliferative index was correlated with age higher than 50y (227), and with high mortality of the patients with PTC (5, 149). These results suggest that proliferative index could help to recognize clinically aggressive PTC but this assumption remains unproven due to the existence of many contradictory reports on record (for a through review see 58, 59, 85, 149).

At variance with well differentiated carcinomas in general and PTC in particular, some of the less differentiated tumours (poorly differentiated carcinomas) and almost every or every undifferentiated carcinomas, which constitutes the end stage of thyroid cancer, display very high proliferative rates (5, 149, 153). The results we obtained using PCNA and MIB-I antibody in the study of different thyroid lesions enlightens the clear cut difference between these two groups of thyroid carcinomas and fits with their distinct biological behaviour (27, 58, 59, 80, 121).

Papillary carcinomas as evaluated by PCNA and MIB-1 appear as (very) low proliferative lesions. The low proliferative activity of PTC is more evident in the subset of carcinomas presenting rearrangement of ret as evaluated by MIB-1 antibody than in PTC without ret rearrangement. This finding, together with the low proliferative rate and the high lymph node metastatic ability of most PTC, regardless the presence or absence of ret rearrangement, turns the neoplastic progression of PTC a very interesting biological model (see below).

The net growth of a tissue results from the balance between the growth rate and apoptotic death rate. The **bcl-2** gene, first detected as a putative oncogene implicated in human follicular lymphoma, was the first gene shown to be involved in the regulation of apoptosis. Bcl-2 is a negative regulator of cell death that does not appear to play a

unique role in the regulation of apoptosis (72). Whether or not bcl-2 is able to suppress apoptosis following the appropriate stimuli may depend on which other members of bcl-2-family related proteins (bax, bcl-x, bak, mcl-1) are being expressed in the cell (for a review see Hale et al, 1996).

We detected in PTC a tendency to a low expression of bcl-2 when compared to the normal thyroid parenchyma. This lower expression of bcl-2 in PTC than in the normal thyroid was also found by others (16, 128, 151, 153). Pilotti et al, 1994, and Pollina et al, 1996, found a much higher expression of bcl-2 in PTC than in poorly differentiated/ undifferentiated carcinomas and advanced that bcl-2 expression is related to the degree of differentiation of thyroid carcinomas (151, 153).

Basolo et al.,1997, found a very low apoptotic index (AI) in PTC which was inversely correlated with bcl-2 expression and positively correlated with p53 expression (5). Similar data on bcl-2 and p53 expression were reported by others (151, 153). The putative apoptotic pathway of PTC was addressed in this work only through the evaluation of bcl-2 expression. We realize that the immunodetection of bcl-2 is clearly insufficient for the evaluation of the apoptotic rate. However if one takes together the relatively high bcl-2 expression in PTC (lower but much more similar to that of normal thyroid than to the low or no expression at all in poorly differentiated/ undifferentiated carcinomas) with the extremely rare mitotic figures and apoptotic bodies in most PTC, it seems logical to conclude that PTC probably represents a model of cancer in which the cell balance stems from an equilibrium of reduced cell proliferation and reduced cell death.

P53 can trigger cell-cycle growth arrest or the apoptotic pathway in response to DNA damage (72, 120). Furthermore, p53 is a positive regulator of bax expression and a negative regulator of bcl-2 expression (72, 120). High levels of bcl-2 protein favour cell survival, whereas high levels of wild-type p53 lead to an increase in bax and a decrease in bcl-2, pushing towards apoptotic cell death. A positive correlation between apoptotic

index and p53 expression was found in PTC (5), suggesting, that (over)expression of non-mutated p53 could be inducing cell death (see below).

An inverse correlation between bcl-2 and p53 expression is observed in undifferentiated carcinomas of the thyroid (151, 153). Although characterization of p53 and bcl2 expression may be expected to give an indication of the ability for survival or death, it should be noted that undifferentiated carcinomas of the thyroid frequently harbour p53 mutations (39, 91). In other words we should always take in consideration, when evaluating p53 immunoreactivity, that the antibodies used in almost all series on record cannot distinguish between high levels of wild-type p53 that may predispose towards cell death and overexpression of mutant, stabilized, p53 protein that are inactive or even oncogenic.

We did not observe any PTC with p53 overexpression. Taking together the results reported in series on record, p53 immunoreactivity was found in around 12.4% of PTC (68/550). Some authors did not find p53 immunoreactivity at all in PTC (130, 218). Those who reported p53 expression in PTC (37, 68, 86, 143, 145, 151, 153, 163, 165, 170) described usually the immunoreactivity as focal or involving less than 10% of the neoplastic cells (37, 86, 143, 145, 151, 153).

P53 mutations were found in 2.4% of the PTC studied in several series (6/250) (38, 50, 55, 91, 99, 143, 165, 179, 180, 229). The majority of the cases harbouring a p53 mutation (five out six) were in the setting of radiation induced PTC (55, 143).

The figures for p53 immunoreactivity and p53 mutations in PTC, 12.4% and 2.4% respectively, suggest that at least part of the immunoreactive cases are not associated with irreversible gene alteration (and can be due to other causes). Jossart et al., 1996, found intense immunoreactivity for p53 in two PTC cell lines harbouring p53 mutation in contrast to the original tumours, and 34 primary PTC where no p53 mutations were found; in these negative cases for p53 mutation no, or only few cells, showed p53 immunoreactivity. Similar results were reported by Dobashi et al., 1993, and 1994, who did not find p53 mutations in well differentiated carcinomas showing low percentage of

immunoreactive cells in contrast to poorly differentiated carcinomas and undifferentiated carcinomas where extensive immunoreactivity and p53 mutations were frequentely found. Supporting this view, Jossart et al., 1996, advanced that widespread immunohistochemical detection of p53 in thyroid carcinoma is associated with mutation. This observation may be related to the sensitivity of the detection method – a representativity of at least 20% of the cells harbouring mutations being needed to be detectable with the methods usually performed (SSCP and DGGE). Or, the other way around, scattered immunoreactivity for p53 could be related to mechanisms, other than mutation, causing the stabilization of the protein (9, 19, 222). As already mentioned, increased expression of wild-type p53 seen as scattered immunoreactivity, may represent the basis together with the expression of bcl-2 family related genes, of the apoptosis pathway in PTC (5, 153). Our data are in agreement with this since we only found more than 25% of immunoreactive cells in undifferentiated carcinomas. A new method, based on laser microdissection, can contribute to clarify this point by allowing the analysis of p53 mutation at the level of a single immunopositive cell as described for E-cadherin mutations in gastric cancer (7).

P53 mutations in cell lines originated from thyroid carcinomas (PTC, follicular carcinomas and undifferentiated carcinomas) have been found by several groups (48, 51, 91, 99, 165, 218, 221) supporting the hypothesis of Wright et al., 1991 that p53 mutations may facilitate growth and immortalization *in vitro*.

P53 immunoreactivity was found in 61% of cases of the tall cell variant in contrast with a control group of common PTC showing 11% of positive cases (163). In this series, p53 immunoreactivity was associated with an increased rate of local and distant recurrences (163). P53 immunoreactivity and/or p53 mutation was also associated with local recurrence in the series of Nishida et al., 1996, and Fogelfeld et al., 1996. Gerasimov et al., 1995, found p53 positivity in PTC in patients older than 50y with aneuploid tumours, two indicators of poor prognosis (68). Sapi et al., 1995, did not find association between p53 alteration (immunoreactivity or loss of heterozygosity) and metastatic potential in contrast with follicular carcinomas where this association was

found. Pollina et al, 1996, failed to show any prognostic difference, which could be related with p53 expression, within the same histotype. We have studied mainly common (well differentiated) PTC, and therefore we can not rule out the possibility that less differentiated (and more aggressive) PTC may display a higher prevalence of p53 expression/mutation. It is also possible that microwave pretreatment would unmask some scattered p53 positive cells in our series of PTC as described by Holm et al., 1994.

Overexpression of mutant p53 in PTC cell lines has no effect on basal proliferation (8, 222). Wyllie et al. 1995, proposed that "this finding emphasizes that cancer cells can acquire the genetic events needed for malignancy (i.e. invasion and metastasis) without needing to loose either cell-cycle check-point control or p53 functions. Such cells exhibit a rate of DNA damage sufficiently normal to not invoke the growth-inhibitory response mediated by p53 (as a result of which there is no selective pressure for p53 mutation)".

Data on PTC and poorly differentiated/ undifferentiated carcinomas indicate that p53 alterations may contribute to the progression of PTC. It seems that this transition occurs, in PTC, in a dramatic way in contrast to a stepwise process in other tumour models (204). In fact, Matias-Guiu et al, 1994, did not find p53 expression in PTC co-existing with undifferentiated carcinomas. Ito et al., 1993, in three cases of PTC co-existing with undifferentiated carcinoma, found p53 mutation only in the undifferentiated counterparts; one PTC displayed already LOH for p53, as in the undifferentiated carcinoma, adding molecular evidence to the transition of PTC to undifferentiated carcinoma through the p53 mutation. Curiously, the frequency of p53 mutations in undifferentiated carcinomas reported in the different series lies between 30-83% (39, 50, 91). Even considering the different sensitivities of the different methodologies it is tempting to advance that at least in a minority of cases alterations in genes other than p53 can lead to a similar end stage of thyroid tumour development. Cyclin kinase inhibitors, as p16 and WAF1/p21, which were found alterated in thyroid cell lines and/or primary tumours (98, 196) appear to be good candidates. However, p53 has been until now the only gene undoubtly and consistently involved in the

progression of PTC, namely in the cases of PTC coexisting with undifferentiated carcinoma (see also point 4).

Genetic instability evaluated as microsatellite instability (MI) seems to be a restricted phenomenon in thyroid lesions. We have found instability in some benign and malignant lesions, without any particular concentration in the papillary subtype; only a minority of cases correspond, however, to the "true" mutator phenotype (meaning by this "classification" cases with a high number of involved microsatellite loci). In the only PTC that fitted with this phenotype, as well as in all the cases showing MI, no alterations of the mismatch repair gene hMSH2 were identified. The possibility of involvement of other mismatch repair genes can not be obviously ruled out but does not seem likely since thyroid carcinomas do not form part of the spectrum of HNPCC (see below). The cases presenting MI at a single locus may reflect the influence of other phenomena, namely polymerase saturation (due to a constant stimulus for proliferation) and/or a higher oxidative stress. This latter hypothesis makes sense in thyroid due to the existence of a physiologic oxidative system resulting from TPO activity but, as previously referred, TPO seems to be down-regulated at the transcriptional level in PTC. It will be interesting to evaluate the proliferation rate and the expression of TPO in thyroid lesions presenting MI. It is also crucial to look for the integrity of repair genes other than hMSH2 in thyroid lesions exhibiting a mutator phenotype.

The putative existence of a mutator phenotype in thyroid lesions, particularly in PTC,might be considered taking in consideration the similarity of the clinicopathologic characteristics identified in tumours of the HNPCC spectrum (where a mutator mechanism of carcinogenesis does usually occur) and those of PTC. In fact, MI was found in colorectal tumours which are frequently diploid, display rare genetic alterations and carry good prognosis (150, 173), features shared by PTC. Sporadic gastric tumours showing MI share also some of these features (173). The lack of any known involvement of thyroid tumours in patients with HNPCC, the few cases of our study showing MI at multiple loci and the absence of alterations in the hMSH2 repair

gene, suggest that this mechanism is not very operative in thyroid lesions being, most likely, a minor event in thyroid carcinogenesis. Since we detected MI both in benign and malignant lesions we have advanced the hypothesis that, when present, MI is likely to occur as an early step of thyroid tumourigenesis.

The existence of two distinct pathways leading to cancer – "suppressor" and "mutator" – is now becoming increasingly accepted. This is due to the emergence of clear differences in the spectra of cancer genes alterated in the two groups of tumours. Generally, aneuploid cancers follow the suppressor pathway and carry a diverse spectrum of mutations in tumour-suppressor genes such as p53 and APC. Tumours of the mutator phenotype, on the other hand, are usualy diploid and exhibit mutations in mismatch repair genes leading to frameshifts in hot spots for slippage induced mutations in "target genes", such as IGFII-R or TGFβR (150).

If one goes back to the question raised in the Aims: "Is any of the two mechanisms of carcinogenesis, suppressor or mutator, operating in thyroid tumours, as proposed for other organs?" it seems logical to conclude that none of these two main mechanisms can be, as seen from the data on record and our own, directly ascribed to PTC.

In fact, mutations of p53 tumour suppressor gene are extremely rare in PTC (see above). APC mutations were also not found in sporadic PTC (26, 28, 232) although an association between familial adenomatous polyposis (FAP) and (papillary) thyroid carcinoma is well documented (74, 83). Some authors claim that FAP associated-thyroid tumours can be considered as a separate entity of follicular cell neoplasm (74, 83), though recently ret rearrangement was found in two out of three FAP associated carcinomas from an extended kindred (23). As stated before MI occurs rarely in PTC and alterations in repetitive sequences in "target genes" have not been reported in PTC to the best of our knowledge.

In contrast, undifferentiated carcinomas of the thyroid frequently display mutations of p53 tumour suppressor gene and Zeki et al., 1994, found an APC mutation in an undifferentiated cell line and an undifferentiated thyroid carcinoma (232).

Taking in consideration the data on the progression of PTC to less differentiated and undifferentiated forms of thyroid cancer with the high frequency of p53 inactivation, gross genetic alterations and aneuploidy, it is likely that in the more advanced steps of progression inactivation of p53 and/or APC ("suppressor pathway") represents the most frequent mechanism.

Progression in PTC seems to follow a very particular pattern. It is possible that progression *in vivo* results from, as proposed by Wynford-Thomas, 1997, a two-step mechanism: epithelial-mesenchymal differentiation switch and occurrence of p53 mutations. "The rarity with which the transition to undifferentiated cancer is seen clinically in the thyroid can be explained by the need for the differentiation switch and the p53 mutation to arise independently in the same cell before any selective advantage is obtained" (225). The aforementioned rarity of progression from PTC to undifferentiated carcinoma, which is shared by FTC, reflects also the very low proliferation rate of almost all differentiated thyroid carcinomas, turning unlikely the chance of acquiring additional mutations and therefore the chance of progression to undifferentiated carcinomas.

2. Are growth factor receptors specifically deregulated in PTC (or in a subset of PTC) at gene/expression level?

The well known importance of growth factors/ growth factor receptors signalling in thyroid and the reports on the putative involvement of **c-erb B2** in the pathogenesis of PTC led us to study the expression of c-erb B2 in thyroid lesions.

The expression of c-erb B2 in thyroid in general, and in PTC in particular, remains a controversial issue. Lemoine et al., 1990, did not find c-erb B2 expression at all in PTC, whereas specific overexpression of c-erb B2 in PTC was reported by some authors both at the protein and at the mRNA level (2, 77, 79).

Taking the above mentioned reports as the two ends of the spectrum we may conclude that our findings lay somewhere in between. In fact, we did find c-erb B2 expression in

PTC, without observing, however, any particular pattern that could be specifically ascribed to PTC. We observed c-erb B2 cytoplasmic immunoreactivity in every type of (follicular cell) thyroid lesion as well as in normal thyroid. Similar findings were reported by Cameselle-Teijeiro et al., 1997.

Previous reports using frozen sections claim that c-erb B2 immunostaining in PTC is predominantly localized in the membrane (77). Cytoplasmic staining was afterwards reported by Akslen et al., 1995, in 88% of PTC using paraffin embedded sections, whereas membrane staining was found in 52% of the PTC. In our hands, like in those of Cameselle-Teijeiro et al, 1997, and using only paraffin embedded material, the expression of c-erb B2 was always cytoplasmic both in normal thyroid and in the different types of (follicular cell) thyroid lesions. No technical artifact seems to be responsible for this pattern of expression for two reasons: first, we obtained almost identical results with two different antibodies for c-erb B2; secondly, in the other models used as positive controls (carcinomas of the stomach and breast) the utilization of the same monoclonal antibodies disclosed a clear membranous pattern.

Immunohistochemical membranous staining for c-erb B2 has been associated with gene amplification which represents the main type of activation of c-erb B2 proto-oncogene in human tumours. Some authors reported, however, c-erb B2 membranous overexpression without detectable gene amplification (87). By Southern blot we did not find any gross gene alterations (rearrangements or amplification). Similar results were reported by other authors (2, 118). Lemoine et al., 1990, did not also find activating mutations of the transmembrane-encoding region of c-erb B2.

The protein detected in our study (130-140 Kda) differs from the standard molecular weight attributed to c-erb B2 (185 Kda). De Potter et al., 1989, also detected a c-erb B2 protein with a low molecular weight in normal tissues of various organs and in breast lesions; the staining in De Potter et al., 1989, series is, like in ours, clearly cytoplasmic. A similar phenomenon—cytoplasmic expression and a lower than normal molecular

weight of the protein—was reported later on with regard to the tandem **EGFR** and PTC (140). It was advanced that this finding could be due to an increased turnover and degradation of the receptor with subsequent accummulation of a degradation product in the cytoplasm (140). It was suggested, moreover, that this increased turnover might be attributed to a TGFα/EGFR autocrine loop in PTC (140).

Strong cytoplasmic expression of EGFR was associated with extra-thyroidal tumour invasion and found by multivariate analysis to be the strongest independent predictor of recurrent disease in the series of Akslen et al., 1995. The existence of an TGFα/EGFR autocrine loop in neoplastic and non-neoplastic lesions of the thyroid was proposed by Aasland et al., 1990 (1) and Lemoine et al., 1991.(118). Haugen et al. in 1993 claimed that the autocrine loop was particular to PTC since they found higher levels of TGFα and EGFR mRNA in these tumours (78). In contrast to this, Westermark et al., 1996, found similar levels of EGFR mRNA in normal and neoplastic tissues collected from the same thyroid glands. We attempted to evaluate the EGFR expression in thyroid lesions by immunohistochemistry. Immunoreactivity was detected in normal thyroid and in thyroid lesions but the poor quality of the immunohistochemical staining, namely regarding the intense background, did not enable us to quantify accurately this receptor's expression (data not shown).

Neuregulins appeared at first to be c-erb B2 ligands but discordant results were obtained in some cell lines, suggesting the existence of a receptor/co-factor necessary for activation of c-erb B2. It was demonstrated that EGFR couples several agonists (TGFα, EGF and amphiregulin) to other members of the type I family of tyrosine kinase receptors (c-erb B2, c-erb B3 and c-erb B4) (156). This transmodulation of c-erb B2 by the EGFR is biologically relevant since it alters c-erb B2 phosphorylation and stimulates kinase activity (70). The most likely mechanism is that EGF/TGFα stimulates the production of c-erb B2/EGFR heterodimers in which cross-phosphorylation occurs (for a thorough revision see Hynes et al., 1994). It remains to be seen if a "cross-talk" between these receptors exists in normal thyroid tissue and/or in

thyroid tumours; such "cross-talk" could explain the frequent expression of type I family of tyrosine kinase receptors in several thyroid lesions. This holds particularly true for PTC in which coexisting overexpression of EGFR, c-erb B2, c-erb B3 and c-erb B4 has been demonstrated (79).

Table 11 - Comparison of data on EGFR and C-erb B2 expression at the protein and mRNA level in different series of normal thyroid (NT) and papillary thyroid carcinoma (PTC).

	EGFR	NT	EGFR	PTC	c-erb B2	NT	c-erb B2	PTC
Author	immuno	mRNA	immuno	mRNA	immuno	mRNA	immuno	mRNA
Asland,1988		4\4		5\5†*		5\5		8\8†*
Asland,1990		10\10		16\16†				
Lemoine,1990					0\7		0\24	
Song,1991	0\?		18\20					
Lemoine,1991	0\60		24\25					
Haugen,1992					0\5	5\5	12\17	17\17†*
Haugen,1996			54/56				49/56	
Akslen,1995	few cells		120\125				110\125	
Soares,1994					24\25		9\10	
Westermark,1996	4\4	5\5	7\7†*	2\2				
Cameselle-T,1997					7\7		5\5	

† - High levels compared to non-neoplastic tissue * - Including lymph node metastases

The reports on the expression of EGFR and c-erb B2 at the mRNA level in the different series, show constant results concerning detection of the mRNA of these receptors in normal thyroid as well as in thyroid lesions (Table 11). At variance with this relative homogeneity of data, the immunohistochemical detection of both proteins led to more controversial results concerning both the pattern of immunoreactivity (membranous or cytoplasmic) and the type of lesions expressing these receptors. Technical and methodological reasons can partly explain these discrepancies (different antibodies and/or the use of frozen/paraffin embedded material) from series to series. The comparison of the mRNA and protein expression in each series and the systematic absence of structural genetic alterations suggest that EGFR and c-erb B2 can be (and probably are), regulated at the post-translational level in thyroid lesions.

Our finding of a 130-140 Kda c-erb B2 protein remains a puzzling issue. We may be identifying a precursor molecule of the c-erb B2 gene product rather than its usual, final form. Relating the molecular weight of the detected band (130-140 kDa) with the molecular weight (about 137 kDa) of the apoprotein of the c-erb B2 glycoprotein, it is

tempting to suggest that one may be dealing with an unglycosylated form of the c-erb B2 protein. It is, however, hardly conceivable that only a precursor form can be identified in such a wide range of lesions and even in normal parenchyma. As proposed by Ness et al., 1996, with regard to EGFR, cytoplasmic c-erb B2 staining may also reflect a rapid turnover of the c-erb B2 receptor in the thyroid following activation under partial control by EGF\TGFα\EGFR, thus leading to its internalization and degradation.

The expression of c-erb B2 and the distinct molecular weight of its product we found in normal thyroid and in several pathological conditions of the thyroid remains difficult to understand in terms of the dogma on the pathogenetic meaning of c-erb B2 in PTC advanced by Haugen and collaborators (2, 77). According to these authors, and in conflict with our results, PTC show increased expression (at protein and mRNA level) of c-erb B2 suggesting a significant biological mechanism of this receptor system in PTC (77).

In conclusion, Akslen et al., 1995, reported an association between c-erb B2 cytoplasmic expression and the occurrence of PTC in younger patients with a good prognosis; for these authors c-erb B2 immunoexpression may be used as a marker of malignant transformation and low grade histologic aggressiveness rather than of poor prognosis (3). We did not disclose any association of c-erb B2 expression in PTC and the age of patients or other clinicopathological features. Our finding of an ubiquitous and similar expression of c-erb B2 in benign and malignant lesions as well as in normal thyroid, and the absence of any demonstrable gene alterations in our cases of PTC, suggest that c-erb B2 protein may be involved in "normal" functions of thyroid growth and differentiation rather than in neoplastic transformation.

The tyrosine kinase receptor **c-met/HGFR**, which is the receptor for the Hepatocyte Growth Factor (HGF) is involved in human neoplasms by rearrangement, amplification and/or overexpression (195). HGF has been identified as a scatter factor stimulating

dissociation, mobility and proliferation in epithelial cells (190). The HGF was found to be *in vitro* a potent mitogenic factor for normal and oncogene transformed human thyroid cells (45). Dremier et al., 1994, showed that HGF stimulates the proliferation and mobility of dog thyrocytes and represses the expression of differentiation markers (41).

In normal thyroid it was demonstrated the presence of transcription of c-met/HGFR mRNA, but not the immunohistochemical expression of c-met/HGFR protein; unstable protein or inefficient translation were presented as possible explanations for these findings (154).

In our hands, c-met/HGFR was found overexpressed in the majority of PTC; no gross alterations of the gene were found by Southern blot. The putative prognostic implications of the expression of c-met/HGFR are not yet clarified. Di Renzo et al., 1992, found a relationship between over-expression of c-met/HGFR and guarded prognosis, whereas Gangemi et al.,1997, reported the low/absent expression of c-met/HGFR as the single factor most strongly related to the risk of developing hematogeneous metastases in PTC. Absence or reduced expression of c-met/HGFR in poorly differentiated and undifferentiated thyroid carcinomas has been reported (159).

In our series no significant relationship was found between c-met/HGFR overexpression and mitotic index and other clinicopathologic features (age, tumour size, vascular invasion, lymph node involvement), thus not throwing any light on the putative prognostic meaning of c-met/HGFR (over) expression in PTC. Similar results were reported by Rucco et al., 1996.

Overexpression of c-met/HGFR was detected in human follicular cells after introduction of mutant ret or ras genes (93); it was thus concluded that c-met/HGFR overexpression was induced by activation of the signalling pathway of those oncogenes. Since activated oncogene expression in thyroid cells can result in c-met/HGFR overexpression (93), it is

possible that in PTC c-met/HGFR overexpression merely reflects the presence of the activation of one oncogene (ret or another gene). Zanetti et al., 1997, reported c-met/HGFR expression in cell cultures from normal thyroid, remaining unclarified if culture conditions have any effect in c-met/HGFR expression. The expression and activity of HGF need also to be evaluated in thyroid tumours in order to see if the system HGF/c-met/HGFR plays any role in the initiation or progression of PTC.

In adult tissues, intermediate filament proteins are expressed in a cell-type-specific manner (195). The coexpression of cytokeratins and vimentin is restricted to early development, wound healing and certain types of neoplasm. PTC is one of those types of neoplasm (226). In our series vimentin expression was found in 81% of the PTC and nearly absent in adjacent "normal" thyroid. A close relationship was found between the levels of expression of vimentin and those of c-met/HGFR . The weak point of our results on this issue is that we have analysed mainly PTC, so it is hard to be sure whether the aforementioned relationship is a distinctive feature of PTC or also occurs in other thyroid tumours; however c-met/HGFR is rarely expressed in follicular carcinomas (159). Our results are clearly different from those of Viale et al. 1989, who found expression of vimentin in normal thyroid (201). Again, antibody specificity and methodological differences may be in the basis of this discrepancy.

A particular pattern of cytokeratin expression is also associated with PTC, which shows a shift from low to high molecular weight cytokeratin expression (56, 57). Cells that coexpress cytokeratins and vimentin are thought to originate from epithelial cells that dedifferentiate and synthesize vimentin, or from epithelial cells that have differentiated from a primordial mesenchymal cell type. In melanoma and breast cancer, strong evidence favouring a link between keratins (cytokeratin 8 and 18) and vimentin coexpression, changes in integrins and increased invasive ability was found (82). Tsarfaty et al., 1994, showed that the inappropriate expression of c-met/HGFR in mesenchymal cells can lead to the expression of mesenchymal and epithelial markers in cell lines. It is difficult to disclose if c-met/HGFR expression is the cause or a

consequence of some of the special phenotypic features of PTC. Despite this limitation, PTC seems to be a good model to study the putative relationship between overexpression of c-met/HGFR proto-oncogene, aberrant expression of intermediate filaments and invasive/metastatic potential.

The importance of the growth factor/growth factor receptor model in PTC has been recently reinforced by the findings of the group of Williams (188) on the major role played by IGF-I/IGF-IR in a subset of PTC displaying a marked lymphoplasmocytic infiltrate. These authors found that the tumorous lymphocytes infiltrating this subset of PTC produce large amounts of IGF-I and that the neoplastic cells of these cases overexpress receptors for IGF-I. It looks like as if these carcinomas depend upon the presence of lymphocytes to survive and grow. This hypothesis provides also a reasonable explanation for the ability of these PTC to metastasize to regional lymph nodes despite their slow growth potential. The classic concept of lymph node metastization is challenged by this approach to the problem of highly-metastatic-indolent-growing PTC. The neoplastic cells would not invade lymphatics and grow in the lymph nodes because they are intrinsically more aggressive than the neoplastic cells that rest within the primary tumour; they would do it because they find in the lymphatic channels and in the lymph nodes the appropriate growth factors. This fascinating possibility provides a sound explanation for one of the most intriguing problems of thyroid oncology: lymph node metastization does not worsen the prognosis of patients with PTC (58). If a similar relationship is operative in other types of PTC remains to be elucidated. The study of IGF-I/IGF-IR in lymph node metastases of several types of PTC may contribute to clarify this point.

3. Is the particular metastatic pattern of PTC related to alterations in the epithelial-adhesion molecule E-cadherin?

E-cadherin cell adhesion molecule is a crucial component of the cell-cell adhesion system in thyroid (14, 15). A tumour/invasion suppressor role has been attributed to this molecule in experimental models (203). Loss of E-cadherin expression has been

associated with invasiveness and aggressiveness in some tumour models (62). Several types of E-cadherin gene alterations (missense, frameshift and nonsense mutations, deletions and exon skipping) have been reported in some types of stomach (diffuse or isolated cell carcinoma), breast (lobular carcinoma) and gynaecological cancers (97).

PTC show a reduced and heterogeneous immunohistochemical expression of E-cadherin. The study of mRNA expression performed by Brabant et al, 1993, demonstrated also some heterogeneity which roughly correlates with the variable and frequentely down-regulated immunohistochemical expression of E-cadherin. Association between E-cadherin low expression and lymph node involvement was not found in our series; a similar "negative" finding has been reported later on by other authors (171, 205). These results contradict those of Brabant et al., 1993, who found low or undetectable E-cadherin immunoreactivity in relapsing tumours or with metastatic spreading. Scheumman et al., 1995 found a correlation between distant metastases and low E-cadherin expression; no correlation was found by these authors between E-cadherin expression and with lymphatic spread (171, 205). Primary tumours and the corresponding lymph node metastases do not significantly differ regarding the expression of E-cadherin. Taking all these findings together we think there is evidence to conclude that the ability for the colonization of lymph nodes by PTC cells is not directly correlated with down-regulation of E-cadherin (Unfortunately we still ignore most of the mechanisms involved in the nodal metastization of PTC).

Despite the absence of correlation between decreased expression of E-cadherin and lymph node metastases it has been shown that lack of E-cadherin is an adverse prognostic factor in differentiated thyroid carcinoma (205) and correlates with the presence of distante metastases (171). These findings suggest that the lack of E-cadherin in thyroid carcinomas, like in other tumour models, provides the neoplastic cells with relative advantages regarding the ability for local invasiveness and/or distante metastization (178).

At the gene level, only a case of a diffuse sclerosing variant of PTC showed alteration of the E-cadherin gene, a missense mutation. A series of cases of the diffuse sclerosing variant of PTC and of the so-called "mucoepidermoid variant" of PTC carcinomas is now being studied regarding the E-cadherin gene; these cases frequently present phenotypic characteristics compatible with abrogation of the cell adhesion system. Such abrogation may be responsible for the tendency of these variants of PTC to grow diffusely and to give rise to nodal and distant metastases. At some extent, similar features are also attributed to other tumours (e.g. lobular carcinoma of the breast) showing high frequency of E-cadherin inactivation. Our preliminary results point to the possibility that mutations in the E-cadherin gene are a more frequent event in the aforementioned types of PTC which appear to be particularly prone to diffuse growth and invasiveness than in common PTC (unpublished observations).

Our results show that alterations of the E-cadherin gene are infrequent in common PTC, thus supporting the assumption that the reduced and heterogeneous expression of E-cadherin in these carcinomas reflects transcriptional or post-transcriptional modulation rather than structural abnormalities. It is tempting to suggest, furthermore, that such modulation may depend upon alterations in the expression of growth factors/growth factor receptors. Recently obtained evidence supports this assumption. HGF has been demonstrated to phosphorylate β-catenin leading to the disruption of E-cadherin complex and relocalization of the proteins in the cell (178, 190). In thyroid it was demonstrated, that the down-regulation of E-cadherin results from modulation by growth factors (e.g. EGF) as already suggested in other tumour systems (14, 97, 177). TSH stimulates E-cadherin mRNA expression and this fact can have important pratical implications in the TSH-suppressive treatement (14). It is also important to evaluate the "status" of the other components of the E-cadherin cell surface complex in PTC and other thyroid lesions. In the only report on record Zanetti et al., 1997 found normal or even increased expression of β- and α-catenin in PTC (230).

Other types of molecules involved in cell adhesion have also been studied in PTC. This is the case of CD44, a polymorphic family of integral membrane glycoproteins associated with cell matrix adhesion (hyaluronan, heparan sulphate, fibronectin and others matrix constituents), lymphocyte activation and neoplasia. Low expression of CD44 was found in normal thyroid (54). Strong expression of CD44 was found in 97% of the PTC (54). The isoform CD44v6, associated with metastasis in other tumours, was found in PTC (46). Figge et al., 1994, suggested that deregulated CD44v6 could contribute to the ability of PTC cells to metastasize to regional lymph nodes but later on the same group reported the expression of CD44v6 in adenomas and goitres (47). It was also refered that PTC exhibit specific patterns of aberrant alternative CD44 splicing distinguishing them from histologically normal thyroid tissue (46,47). There is not enough evidence – and we have no personal experience on this issue – to evaluate the putative meaning of aberrant CD44 splicing in the peculiar clinicopathologic features of PTC.

Integrins are another group of molecules actively involved in cell matrix interactions (113). In the mature follicular epithelium of the thyroid the adhesion-mediating complexes to laminin-1, 2 and type IV collagen are $\alpha 3\beta 1$, $\alpha v\beta 1$ and $\alpha v\beta 3$ (113, 172). Loss of $\alpha 3\beta 1$ and "de novo" expression of $\alpha 6\beta 4$ were found in carcinomas with clinical and/or histological aggressiveness (172). Like with CD44 – and we do not also have any experience in the study of integrins – there is not enough data to link integrin alterations to PTC characteristics.

The concept of neoplastic cells as a sort of little bags of proteolytic enzymes able to destroy the extracellular matrix proteins as a way of facilitating tumour cells invasion is no longer tenable. It is now widely accepted that in most neoplastic models stromal cells are the great producers of proteolytic enzymes. A good example is Matrix Metalloproteinase-I (MMP-1) gene whose expression was found in the fibrous capsule of PTC and not in the cancer cells (101). In most cancer models the carcinomatous cells appear often to serve as modulators of such proteolytic activity either by the production of enzyme inhibitors or by the regulation of the expression of enzyme receptors. This

holds true regarding the PTC whose cells have been shown to express α1 anti-trypsin (152).

The modulation of the proteolytic/antiproteolytic system in PTC depends also upon the expression of uPAR by the neoplastic cells (34). In the preliminary study we have done on this issue we found a significant relationship between high uPAR expression and vascular invasiveness of PTC. No association was found with the other clinicopathologic features but for a tendency to more intense uPAR staining in tumours greater than 3 cm. Further studies and larger series are needed to clarify the putative association of uPAR and vascular spread in PTC. Increased uPA expression was found in cells treated with antibody against E-cadherin (178); and distribution of uPA from the perinuclear region to the cell surface appears to be modulated partly by E-cadherin (62, 97). We did not evaluate uPA expression, but no correlation was found with regard to E-cadherin and uPAR expression in our series. Given the lower expression of E-cadherin in PTC and the association we found with venous invasion and uPAR, it remains to be seen if uPA-uPAR system is involved in the invasiveness of PTC.

4- What does ret activation mean from a clinical and pathobiological stand point?

Ret fulfills the criteria as a growth factor receptor in nervous system; however in PTC ret is only expressed whenever it is present in its rearranged oncogenic form. Ret gene rearrangements have been found in about 24% of the clinical evident PTC from our series. An attempt was made to characterise these carcinomas. No morphologic features allowed the distinction of ret rearranged PTC from PTC without ret rearrangement, despite a tendency, for an association between ret rearrangement and frank papillary architecture, and the same holds true regarding the majority of the biologic markers we have studied. The only significant epidemiological difference between the two groups of PTC concerned the lower age of the patients harbouring tumours with ret rearrangement. This finding further supports, in our opinion, the association of ret rearrangement and early onset of PTC. This association may indicate a short-cut (single

or few hits) in ret-induced papillary carcinogenesis. This assumption is in part substantiated by experiences in transgenic mice with targeted expression of ret/PTC1 oncogene which developed thyroid carcinomas with considerable similarities to human PTC (96).

Bongarzone et al, 1996 found that the frequency of ret and trk activation is significantly higher in the group of patients aged 4-30 years. Jhiang et al., 1992, in their limited series, found also a significant lower age of the patients presenting PTC with ret rearrangement. The same trend is further supported by the concurrent increase in the incidence of PTC in children after the Chernobyl disaster (141, 142) and by the high frequency of ret activation in these cases (about 60%) (105).

It is difficult to ascertain if age "per se" is a key factor in the development of these tumours. Some authors found that when gender is taken into account no significant differences were found in the prevalence of ret rearrangement in PTC of children and adults (214, 215). Based upon their findings of a relationship between gender and ret rearrangements Williams GH et al., 1996, suggested that ret gene could interact with hormonally dependent mechanisms.

The finding of Viglietto et al.,1995, and Santoro et al.,1996, of a much higher frequency of ret rearrangement in papillary microcarcinomas than in clinically evident PTC has been advanced as an extra argument favouring the association between ret rearrangement and initiation and early onset of PTC. This reasoning is based upon the assumption that microcarcinomas represent recently developed PTC. We do not question the existence of an association between ret rearrangement and initiation of PTC but we disagree with the concept that papillary microcarcinomas should necessarily represent recently developed neoplasms. As a matter of fact we think that most microcarcinomas are probably as "old" as common PTC. In fact if one compares the extremely high prevalence of papillary microcarcinomas at autopsy and in normal appearing surgical specimens removed together with benign thyroid lesions, with the

low incidence of clinically evident PTC, one has to conclude that most of these carcinomas, once originated in the thyroid gland, probably remain as small lesions (182, 216).

Sobrinho-Simões et al., 1979, as well as Komorowski and Hanson, 1988, showed that older patients have a higher prevalence of latent papillary microcarcinomas whereas the age-specific prevalence curves described by Sampson et al., 1969, in Hiroshima and Nagasaki showed a peak about 35-45 years. The linear pattern of age specific prevalence observed in some series of autopsy cases is suggestive, following the model of Knudson et al., 1973, that only the first "hit" of carcinogenesis has occurred in latent papillary microcarcinomas; this finding suggests, furthermore, according to the so-called two step theory of cancer development, that these carcinomas, once originated, might have a deficient growth promotion (182) due to either the lack of (circulating and/or locally produced) growth promoting factors or the irresponsiveness of neoplastic cells to the usual growth stimuli. The same explanation holds (basically) true if one follows the data on occult microcarcinomas of the series of Harach and Fransilla, 1987. These authors found that the prevalence of such microcarcinomas did not increases much in adult life and was similar in men and women; these observations fit with the possibility of papillary microcarcinomas progressing only very rarely to clinically evident PTC and support the assumption that sex hormones or sex related growth factors may be the promoting factors involved in such progression

The trend to a lower proliferative rate in ret rearranged PTC detected in the present study fits also with the findings of Viglietto et al., 1995, and Santoro et al.,1996, on the association between papillary microcarcinomas and ret rearrangement, as well as with the findings of Bond et al., 1994, of a poorer growth potential *in vitro* of thyrocytes transformed with amphotropic vectors carrying activated ret when compared for example with ras activated cells. Our series is however too small to study the putative relationship between the size of the tumours and the presence of ret rearrangement; it is particularly inadequate to address the issue of papillary microcarcinomas, which

occur mostly as incidental finding in thyroidectomy specimens for benign conditions or at autopsy, since our series is composed of clinically evident PTC. For their extremely small size and frequent detection either in histological slides after paraffin embedding of benign lesions or at the autopsy, any epidemiological study on papillary microcarcinomas should rely, as Viglietto et al.,1995, did in their series, on the immunohistochemical detection of ret activation rather than on a Southern blot approach. Unfortunately reliable anti-ret antibodies working in paraffin sections were not commercially available and this approach was not possible.

It is not easy to reconciliate some of our findings with the aforementioned association between papillary microcarcinomas and ret rearrangement. For instance, cases showing ret rearrangement occurred in younger patients and displayed a suggestively lower proliferative rate than common PTC; despite this, their size was similar to those of PTC without rearrangement. It is obviously impossible to go in depth in the discussion of this apparent contradiction because it is not known the time mediating between the genesis of the tumours and the clinical manifestation of the disease. It would be possible, for example that PTC with ret rearrangement in our series may represent a subset of "old" PTC; this assumption would be in accordance with an early age of onset and their very low proliferative rate. Or, alternatively, PTC may present distinct levels of apoptosis which would be smaller in tumours with ret-rearrangement than in PTC without rearrangement. We did not evaluate the apoptotic rate and we have not therefore any definitive conclusion on this issue despite the similar level of expression of bcl-2 in PTC with and without rearrangement.

Other possibility is the existence of age specific factors which might be particularly important in PTC with and/or without ret-rearrangement. One of such a factors could be the IGF- I. We have already discussed the importance of locally produced IGF-I in the promotion of a subset of PTC displaying abundant lymphoplasmocytic infiltrate. We have also previously addressed the so-called IGF-I autocrine circuit induced by TSH. It is also known that growth hormone increase IGF-I local levels in target organs (17). We

will concentrate now on the possibility that circulating levels of IGF-I may play a pathogenic role in ret rearranged PTC.

There is a long-life fluctuation of total IGF-I levels which peaks in the adolescence (12-16y) and is more pronounced in females than in males (111). Free IGF-I peaks in the early infancy and the ratio free/total IGF-I is maintained at a higher level in prepubertal children (76). It was demonstrated that the ret/PTC2 truncated protein binds specifically to the enigma transducer protein of the Insulin receptor pathway and that this binding is necessary for the mitogenic activity of ret/PTC2 (44, 220). If one admits IGF-I may as well activate enigma protein it is possible to assume that circulating IGF-I can be important for PTC growth in the presence of at least the ret/PTC2 rearrangement. This hypothesis fits with the gender (M:F=1:7) and age (28.1 y versus 44.7y) of clinically evident ret rearranged PTC (Table 9).

It remains to be found whether the apparent greater clinical aggressiveness of the so-called post-Chernobyl PTC (141, 142) may reflect the influence of other types of ret rearrangement (63, 105, 213), or the co-existence in this setting of other (age related) factors putatively involved in cancer development. Ras, p53 and Gsα mutations seems to be uncommon in the PTC occurring in this setting (143, 207) as in PTC in general.

If one adheres to the concept that most papillary microcarcinomas are not recently developed PTC, the high prevalence of ret rearrangement in these carcinomas as reported by Viglietto et al., 1995, and Santoro et al., 1996, becomes very interesting because it supports the hypothesis advanced by Viglietto et al., 1995, that tumours harbouring a ret/PTC activation could proceed less frequentely towards fully developed PTC than papillary tumours lacking ret rearrangement. This hypothesis fits also with the findings of Bond et al., 1994, of a poorer growth potential *in vitro* of thyrocytes transformed with activated ret when compared with thyrocytes transformed with activated ras (11).

Other types of evidence reinforce the assumption that most ret rearranged PTC do not tend to progress towards more advanced steps of neoplastic development. Several groups did not find ret rearrangements in less differentiated thyroid tumours (167) despite the frequent occurrence of foci of PTC in poorly differentiated and undifferentiated carcinomas (90, 130). This suggests that tumours harbouring ret rearrangements do not usually evolve to undifferentiated phenotypes (167, 169) or if they do, they loose the rearrangement throughout the process.

In the ret-rearranged PTC we observed lower cytoplasmatic staining for E-cadherin than in the PTC without ret rearrangement. Cytoplasmatic expression of E-cadherin has been associated with diverse forms of impairment of E-cadherin cell adhesion complex (β-catenin inactivation, mutations, abnormal phosphorylation) (97, 178). Serini et al., 1996, found in thyroid carcinomas, in contrast to normal thyroid and adenomas, a pericellular distribution of E-cadherin; this distribution was associated with decreased tyrosine phosphorylation and loss of detergent insolubility (172). Serini et al., 1996, suggest that tyrosine phosphorylation of E-cadherin may control the structure of the molecule, and, hence intercellular adhesion, as well as its stabillity within the membrane plane (172). The dissociation from junctional regions could drive the E-cadherin complex away from kinases and phosphatases responsible for the phosphorylation status of the molecule under normal conditions. In this respect, ret rearranged PTC seems to present a lower redistribution of the complex, but further studies are needed to evaluate E-cadherin phosphorylation status and β- and α-catenin expression. As discussed above, it has been advanced that colonization of lymph nodes by PTC cells is not mainly correlated with down-regulation in E-cadherin; it is not therefore contradictory that ret rearranged cases give often rise to lymph node metastases despite presenting probably a more conserved E-cadherin adhesion system than PTC without ret-rearrangement (for a more detailed description of the alterations of the E-cadherin complex see also point 3).

Models of papillary tumourigenesis

PTC present increased expression of various growth factors and growth factor receptors. In several models a role in cell transformation was attributed to increased growth factor stimulation. The absence of apparent genetic abnormalities in growth factors/growth factor receptors in PTC suggests that at least to some extent epigenetic events leading to overexpression of growth factor/growth factor receptors may play a role in the neoplastic initiation of follicular cells. Dawson et al. 1995, using IGF-I autocrine secretion in thyroid cells as an example, suggested that a field effect must exist to explain carcinogenesis in this context. According to Dawson et al. 1995, the autocrine overexpression *per se* (even in the background of a genetic alteration leading to the overexpression) is a weak factor unless a physiologic stimulator for growth (e.g. TSH) is present (30).

Ret, trk and ras have been identified as transforming genes in PTC. Altogether they account for around 50% of the genetic events found in sporadic PTC. A feature which seems to be common to every PTC phenotype is the frequent (over) expression of growth factor/growth factor receptors no matter the activating genetic event, if any, underneath the malignant transformation of the follicular cell. This is the case of the reported increased expression of EGFR, TGFα and/or c-met/HGFR and the more disputable (over) expression of c-erb B2 . The same holds true, though not necessarily in terms of increased expression, regarding adhesion molecules and molecules related to invasiveness such as vimentin, CD44, integrins and uPA/uPAR. In fact, abnormal expression of the aforementioned molecules has been reported in PTC without demonstrable abnormalities at the genetic level.

Our results support the assumption that (gross) genetic alterations are not usually underneath the abnormal expression of several growth factors/growth factors receptors and of several adhesion molecules in PTC.

We found ret rearrangements in 24.2% of the PTC in our series. The cases with rearrangement occurred in significantly younger patients than PTC whithout ret rearrangement. The former displayed also a trend to a more marked papillary architecture, a lower proliferative rate and a lower cytoplasmic expression of E-cadherin than the latter.

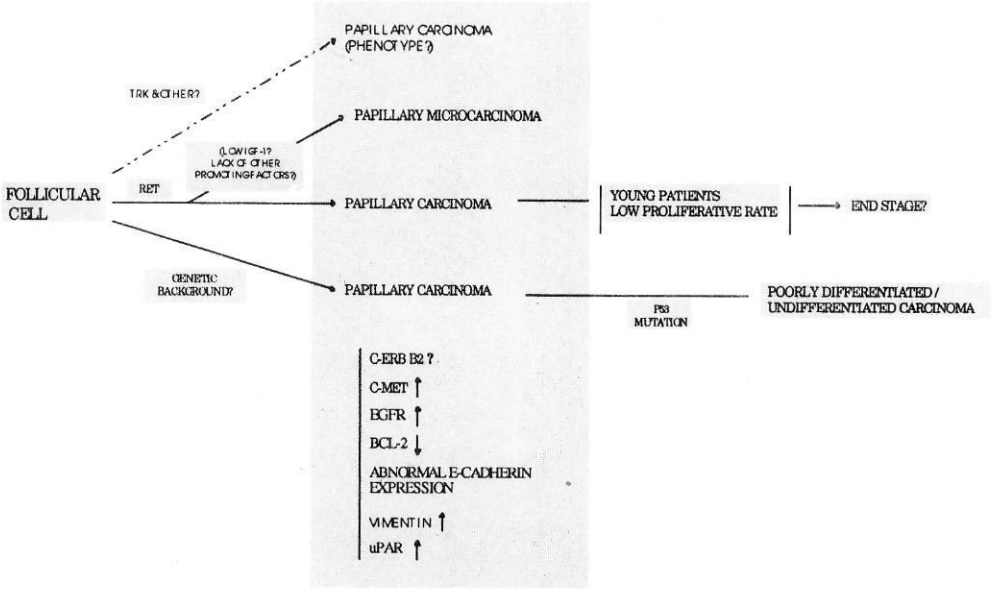
We did not disclose any significant difference in the expression of growth factor/ growth factor receptors (c-erb B2, c-met/HGFR) and other biological markers (vimentin, bcl 2 and uPAR) in cases with and whithout ret rearrangement but our series is too small and the studies not sufficiently exhaustive to allow drawing definitive conclusions on these issues.

Expression of p53 was not found in PTC (with or without ret rearrangement). Genetic instability in dinucleotide microsatellites was found only in a minority of PTC and mutations of the repair gene hMSH2 were not found in any PTC of our series.

When we analyse our data together with the data reported from other groups, two interesting points stand up: a) ret rearrangement seems to be more frequent in papillary microcarcinomas than in clinically evident PTC and, b) nobody has ever demonstrated the occurrence of poorly differentiated or undifferentiated thyroid carcinoma on a background of ret-rearranged PTC at variance with the progression towards less differentiated carcinomas of PTC without ret rearrangement. In the few studies where solid clinicopathologic correlations were performed ret rearrangement was not associated with any of the aggressive variants of PTC. It remains to be clarified the role played by E-cadherin alterations at the genetic and/or protein level in such clinically aggressive variants.

Our results, lead us to propose the existence of two possible routes of PTC origin and progression.

One route in which the most frequent and consistent findings appear to be alteration/deregulation in the expression of growth factors/growth factor receptors; in this route no consistent genetic alterations are found (yet?); this "type" of PTC arises later in life and can progress to less differentiated forms through p53 mutations (schematic drawing). The other route involving ret rearrangement possibly in the setting of a specific age related environment leading frequently to tiny, small growing, quite harmless tumours not evolving to less differentiated forms (as a sort of Bonsai phenomenon) in tumour biology (schematic drawing).



Schematic drawing: models of papillary tumourigenesis (for details see text)

REFERENCES

1 Aasland R, Akslen LA, Varhaug JE, Lillehaug JR: Co-expression of the genes encoding transforming growth factor-alpha and its receptor in papillary carcinomas of the thyroid. Int J Cancer 46: 382-387, 1990.

2 Aasland R, Lillehaug J, Male R et al: Expression of oncogenes in thyroid tumors: co-expression of c-erb B2/neu and c-erb B. Br J Cancer 57:358-363, 1988.

3 Akslen LA, Varhaug JE: Oncoproteins and tumour progression in papillary thyroid carcinoma. Cancer 76:1643-1654, 1995.

4 Basolo F, Pinchera A, Fugazzola L et al: Expression of p21 ras protein as a prognostic factor in papillary thyroid cancer. Eur J Cancer 30A:171-174, 1994.

5 Basolo F, Pollina L, Fontanini G et al: Apoptosis and proliferation in thyroid carcinoma: correlation with bcl-2 and p53 protein expression. Br J Cancer 75:537-541, 1997.

6 Bechtner G, Schopohl D, Rafferzeder M, Gärtner R, Welsch U: Stimulation of thyroid cell proliferation by epidermal growth factor is different from cell growth induced by thyrotropin or insulin-like growth factor I. Eur J Endocrinol 134:639-648, 1996.

7 Becker I, Becker K-F, Röhrl MH et al: Single-cell mutation analysis of tumors from stained histologic slides. Lab Invest 75:801-807, 1996.

8 Blaydes JP, Schlumberger M, Wynford-Thomas D, Wyllie FS: Interaction between p53 and TGFβ1 in control of epithelial cell proliferation. Oncogene 10:307-317, 1995.

9 Blaydes JP, Wynford-Thomas D: Loss of responsiveness to transforming growth factor ß (TGFß) is tightly linked to tumorigenicity in a model of thyroid tumour progression. Int J Cancer 65:525-530, 1996.

10 Bond JA, Ness GO, Rowson J et al: Spontaneous de-differentiation correlates with extended lifespan in transformed thyroid epithelial cells: An epigenetic mechanism of tumour progression? Int J Cancer 67:563-572, 1996.

11 Bond JA, Wyllie FS, Rowson J, Radulescu A, Wynford-Thomas D: *IN VITRO* reconstruction of tumour initiation in a human epithelium. Oncogene 9:281-290, 1994.

12 Bongarzone I, Fugazzola L, Vigneri P et al: Age related activation of the tyrosine kinase receptor proto-oncogenes ret and NTRK1 in papillary thyroid carcinoma. J Clin Endocrinol 81: 2006-2009, 1996.

13 Bongarzone I, Pierotti MA, Monzini N et al: High frequency of activation of tyrosine kinase oncogenes in human papillary thyroid carcinoma. Oncogene 4:1457-1462, 1989.

14 Brabant G, Hoang-Vu C, Behrends J et al: Regulation of the cell-cell adhesion protein, E-cadherin, in dog and human thyrocytes *in vitro*. Endocrinology 136:3113-3119, 1995.

15 Brabant G, Hoang-Vu C, Cetin Y, et al: E-cadherin: a differentiation marker in thyroid malignancies. Cancer Res 53: 4987-4993, 1993.

16 Branet F, Caron P, Camallieres M, Selves J, Brousset P: Expression du protooncogéne bcl-2 dans le tissu thyroidien néoplasique et non néoplasique. Bull Cancer 83:213-217, 1996.

17 Brook C, Marshall N: Essential Endocrinology, 3rd edition. Blackwell Science, Oxford, 1996.

18 Burkitt HG, Young B, Heath JW: Wheater 's Funtional Histology. A text and colour atlas. pp. 310-312. 3rd edition, Churchil Livingstone, Edinburgh, 1993.

19 Burns JS, Blaydes JP, Wright PA et al: Stepwise transformation of primary thyroid epithelial cells by a mutant Ha-ras oncogene: An in vitro model of tumor progression. Mol Carcinogen 6:129-139, 1992.

20 Calabro V, Strazzullo M, La Mantia G et al: Status and expression of the p16INK4 gene in human thyroid tumors and thyroid-tumor cell lines. Int J Cancer 67:29-34, 1996.

21 Cameselle-Teijeiro J, Cameselle-Teijeiro JF, Moldes J, Alfonsin N, Forteza J: Expression of C-erb B2 protein in benign and malignant tumours originating from the follicular epithelium of the thyroid gland. Pathol Res Pract 193: 389 ,1997 (Abstract).

22 Carcangiu M, Zampi G, Pupi A, Castagnoli A, Rosai J: Papillary carcinoma of the thyroid. A clinicopathologic study of 241 cases treated at the University of Florence, Italy. Cancer 55: 805-828, 1985.

23 Cetta F, Toti P, Petracci M et al: Thyroid carcinoma associated with familial adenomatous polyposis. Histopathology 31:231-236, 1997.

24 Cheatham B, Karn R: Insulin Action and the insulin signaling network. Endocr Rev 16:117-141, 1995.

25 Civitareale D, Lonigro R, Sinclair AJ, Di Lauro R: A tyroid-specific nuclear protein essential for tissue-specific expression of the thyroglobulin promoter. EMBO 8: 2537-2542, 1989.

26 Colleta G, Sciacchitano S, Palmirotta R et al: Analysis of adenomatous polyposis coli gene in thyroid tumours. Br J Cancer 70:1085-1088, 1994.

27 Correa P, Chen V: Endocrine gland cancer. Cancer 75: 338-352, 1995.

28 Curtis L, Wyllie AH, Shaw JJ et al: Evidence against involvement of APC mutation in papillary thyroid carcinoma. Eur J Cancer 30A:984-987, 1994.

29 Darnell J Edt : Molecular Cell Biology. 3d Ed. Scientific American Books, New York, 1996.

30 Dawson T, Wynford-Thomas D: Does autocrine growth factor secretion form part of a mechanism which paradoxically protects against tumour development? Br J Cancer 71:1136-1141, 1995.

31 Dawson TP, Radulescu A, Wynford-Thomas D: Expression of mutant p21ras induces insulin-like growth factor 1 secretion in thyroid epithelial cells. Cancer Res 55:915-920, 1995.

32 De Micco C, Ruf J, Chrestian MA et al: Immunohistochemical study of thyroid peroxidase in normal, hyperplastic, and neoplastic human thyroid tissues. Cancer 67:3036-3041, 1991.

33 De Potter CR, Van Daele S, Van De Vijver MJ, et al: The expression of the *neu* oncogene product in breast lesions and in normal fetal and adult human tissues. Histopathology 15: 351-362, 1989.

34 de Vries TJ, van Muijen G, Ruiter DJ: The plasminogen activaction system in tumour invasion and metastasis. Pathol Update 3: 72-87, 1996.

35 Dhanasekaran N, Heasley LE, Johnson GL: G protein-coupled receptor systems involved in cell growth and oncogenesis. Endocr Rev 16:259-270, 1995.

36 Di Renzo MF, Olivero M, Ferro S et al: Overexpression of the c- MET/HGF receptor gene in human thyroid carcinomas. Oncogene 7:2549-2553, 1992.

37 Dobashi Y, Sakamoto A, Sugimura H et al: Overexpression of p53 as a possible prognostic factor in human thyroid carcinoma. Am J Surg Pathol 17:375-381, 1993.

38 Dobashi Y, Sugimura H, Sakamoto A et al: Stepwise participation of p53 gene mutation during dedifferentiation of human thyroid carcinomas. Diagn Mol Pathol 3: 9-14, 1994.

39 Donghi R, Longoni A, Pilotti et al: Gene p53 mutations are restricted to poorly differentiated and undifferentiated carcinomas of the thyroid gland. J Clin Invest 91:1753-1760, 1993.

40 Donghi R, Sozzi G, Pierotti MA et al: The oncogene associated with human papillary thyroid carcinoma (PTC) is assigned to chromosome 10q11-q12 in the same region as multiple endocrine neoplasia type 2A (MEN 2A). Oncogene 4:521-523, 1989.

41 Dremier S, Taton M, Coulonval K, Nakamura T, Matsumoto K, Dumont JE: Mitogenic, dedifferentiating and scattering effects of hepatocyte growth factor on dog thyroid cells. Endocrinology 135:135-140, 1994.

42 Duh Q-Y, Grossman RF: Thyroid growth factors, signal transduction pathways and oncogenes. Endoc Surg 75:421-437, 1995.

43 Dumont JE, Maenhaut C, Pirson I, Baptist M, Roger PP: Growth factors controlling the thyroid gland. Baillière's Clin Endocrin Metab 5:727-754, 1991.

44 Durick K, Wu RY, Gill GN, Taylor SS: Mitogenic signaling by Ret/PTC2 requires association with enigma via a LIM domain. J Biol Chemist 271: 12691-12694, 1996.

45 Eccles N, Ivan M, Wynford-Thomas D: Mitogenic stimulation of normal and oncogene-transformed human thyroid epithelial cells by hepatocyte growth factor. Mol cell Endocrinol 117:247-251, 1996.

46 Ermak G, Gerasimov G, Troshina K, et al: Deregulated alternative splicing of CD44 messenger RNA transcripts in neoplastic and nonneoplastic lesions of the human thyroid. Cancer Res 55: 4594-4598, 1995.

47 Ermak G. Jennings T, Robinson L, Ross JS, Figge J: Restricted patterns of CD44 variant exon expression in human papillary thyroid carcinoma. Cancer Res 56:1037-1042, 1996.

48 Fabien N, Fusco A, Santoro M et al: Description of a human papillary thyroid carcinoma cell line. Cancer 73: 2206-2212, 1994.

49 Fabien N, Paulin C, Santoro M et al: Detection of RET oncogene activation in human papillary thyroid carcinomas by in-situ hybridisation. Br J Cancer 66:1094-1098, 1992.

50 Fagin JA, Matsuo K, Karmakar A et al: High prevalence of mutation of p53 gene in poorly differentiated human thyroid carcinomas. J Clin Invest 91:179-184, 1993.

51 Fagin JA, Tang SH, Zeki K, et al: Reexpression of thyroid peroxidase in a derivative of an undifferentiated thyroid carcinoma cell line by introduction of wild-type p53. Cancer Res 56: 765-771, 1996.

52 Farid NR, Shi Y, Zou M: Molecular basis of thyroid cancer. Endocr Rev 15: 202-232, 1994

53 Fawcet DW, Raviola E, Bloom, Fawcett A: Textbook of Histology, pp.490-497, 12th edition, Chapman & Hall, London, 1993 .

54 Figge J, del Rosario AD, Gerasimov G et al: Preferential expression of the cell adhesion molecule CD44 in papillary thyroid carcinoma. Exp Mol Pathol 61:203-211, 1994.

55 Fogelfeld L, Bauer T, Schneider A, Swartz J, Zitman R: p53 gene mutations in radiation-induced thyroid cancer. J Clin Endocrinol Metab 81: 3039-3044, 1996.

56 Fonseca E, Nesland JM, Hoie J, Sobrinho-Simões M: Pattern of expression of intermediate cytokeratin filaments in the thyroid gland: an immunohistochemical study of simple and stratified epithelial-type cytokeratins. Virchows Archiv 430:239-245, 1997.

57 Fonseca E, Nesland JM, Sobrinho-Simões M: Expression of stratified epithelial-type cytokeratins in hyalinizing trabecular adenomas supports their relationship with papillary carcinomas of the thyroid. Histopathology 31:330-35, 1997.

58 Fonseca E, Soares P, Sobrinho-Simões M: The metastatic pattern of thyroid tumours. A review with an emphasis on the peculiarities of papillary carcinoma. Pathology Update 4: 202-223, 1997

59 Fonseca E, Soares P, Rossi S, Sobrinho-Simões M: Prognostic factors in thyroid carcinomas. Published in german in: Pathologe 18: 275-285, 1997.

60 Fonseca E,Sobrinho-Simões M: Diagnostic problems in differentiated carcinomas of the thyroid. Pathol Res Pract 191: 318-331, 1995.

61 Frauman AG, Moses AC: Oncogenes and growth factors in thyroid carcinogenesis. Endocr Metab Clin North Am 19:479-493, 1990.

62 Frixen UH, Behrens J, Sachs M, et al: E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. J Cell Biol 113: 173-185, 1991.

63 Fuggazola L, Pilotti S, Pinchera A, et al: Oncogenic rearrangements of the RET proto-oncogene in papillary thyroid carcinomas from children exposed to the Chernobyl nuclear accident. Cancer Res 55: 5617-5620, 1995.

64 Fusco A, Grieco M, Santoro M et al: A new oncogene in human thyroid papillary carcinomas and their lymph-nodal metastases. Nature 328:170-173, 1987

65 Fuher D, Holzapfel HP, Wonerow P, Scherbaum WA, Paschke R: Somatic mutations in the thyrotropin receptor gene and not in the Gs alpha protein gene in 31 toxic thyroid nodules. J Clin Endocrinol Metab 82:3885-3889, 1997

66 Gangemi BA, Costantino A, Russo G et al: Negative/low expression of the Met/hepatocyte growth factor receptor identifies papillary thyroid carcinomas with high risk of distant metastases. J Clin Endocrinol Metab 82:2322-2328, 1997.

67 Gartner R: Growth factors in thyroid cells. *In* Current topics in pathology - Thyroid Pathology. Schmid and Bocker (Eds.) 91, 65-81, 1997.

68 Gerasimov G, Bronstein M, Troshina M, et al: Nuclear p53 immunoreactivity in papillary thyroid cancers is associated with two established indicators of poor prognosis. Exp Mol Pathol 62: 52-62, 1995.

69 Goretzki PE, Lyons J, Stacy-Philipps S et al: Mutational activation of ras and GSP oncogenes in differentiated thyroid cancer and their biological implications. World J Surg 16:576-582, 1992.

70 Graus-Porta D, Beerli R, Hynes N: Single-chain antibody-mediated intracellular retention of ErbB-2 impairs Neu differentiation factor and epidermal growth factor signaling. Mol Cell Biol 15: 1182-1191, 1995.

71 Grieco M, Santoro M, Berlingieri MT et al: PTC is a novel rearranged form of the ret proto-oncogene and is frequently detected in vivo in human thyroid papillary carcinomas. Cell 60:557-563, 1990.

72 Hale A, Smith C, Sutherland L et al: Apoptosis: molecular regulation of cell death. Eur J Biochem 236: 1-26, 1996.

73 Hara H, Fulton N, Yashiro T, et al: N-ras mutation: an independent prognostic factor for aggressiveness of papillary thyroid carcinoma. Surgery 116:1010-1016, 1994.

74 Harach HR, Williams GT, Williams ED: Familial adenomatous polyposis associated thyroid carcinoma: a distinct type of follicular cell neoplasm. Histopathology 25: 549-561, 1994.

75 Harach HR, Franssila KO, Wasenius VM: Occult papillary thyroid carcinoma of the thyroid. A "normal" finding in Finland. A systematic autopsy study. Cancer 56: 531-538, 1987

76 Hasegawa Y, Hasegawa T, Fujii K et al: High ratios of free to total insulin-like growth factor-I in early infancy. J Clin Endocrinol Metab 82:156-158, 1997.

77 Haugen D, Akslen L, Varhaug J, Lillehaug J: Expression of c-erb B2 protein in papillary thyroid carcinomas. Br J Cancer 65:832-837, 1992.

78 Haugen DR, Akslen LA, Varhaug JE, Lillehaug JR: Demonstration of a TGF-alpha - EGF-receptor autocrine loop and c-myc protein over-expression in papillary thyroid carcinomas. Int J Cancer 55:37-43, 1993.

79 Haugen DRF, Akslen LA, Varhaug JE, Lillehaug JR: Expression of c-erbB3 and c-erbB-4 proteins in papillary thyroid carcinomas. Cancer Res 56:1184-1188, 1996.

80 Hay ID: Papillary thyroid carcinoma. Clin Endocrinol Metab North Am 19:545-576, 1990.

81 Hedinger C, Williams ED, Sobin LH, (eds.): Histologic typing of thyroid tumours, 2nd ed. World Health Organization — International Histologic Classification of Tumors. Springer Verlag, Berlin, 1988.

82 Hendrix M, Seftor E, Chu Y-W, Trevor K, Seftor R: Role of intermediate filaments in migration, invasion and metastasis. Cancer Metas Rev 15:507-525, 1996.

83 Hizawa K, Iida M, Yao T et al: Association between thyroid cancer of cribriform variant and familial adenomatous polyposis. J Clin Pathol 49:611-613, 1995.

84 Ho Y, Tseng C, chin T, Hsieh L, Lin JD: p53 gene mutation in thyroid cancer. Cancer Lett 103:57-63, 1996.

85 Hofstadter F, Knuchel R, Ruschoff J: Cell proliferation assessment in oncology. Virchows Arch 427:323-341, 1995.

86 Holm R, Nesland JM: Retinoblastoma and p53 tumor suppressor gene protein expression in carcinomas of the thyroid gland. J Pathol 172:267-272, 1994.

87 Hynes NE, Stern DF: The biology of erbB-2/neu/HER-2 and its role in cancer. Biochim Biophys Acta 1198:165-184, 1994.

88 Ishizaka Y, Itoh F, Tahira T et al: Human ret proto-oncogene mapped to chromosome 10q11.2. Oncogene 4:1519-1521, 1989.

89 Ishizaka Y, Shima H, Sugimura T, Nagao M: Detection of phosphorylated ret ^{TPC} oncogene product in cytoplasm. Oncogene 7:1441-1444, 1992.

90 Ito T, Seyama T, Mizuno T et al: Genetic alterations in thyroid tumor progression: association with p53 gene mutations. Jpn J Cancer Res 84:526-531, 1993.

91 Ito T, Seyama T, Mizuno T et al: Unique association of p53 mutation with undifferentiated but not differentiated carcinomas of the thyroid gland. Cancer Res 52:1369-1371, 1992.

92 Ito Y, Kobayashi T, Takeda T et al: Expression of p21(WAF1/CIP1) protein in clinical thyroid tissues. Br J Cancer 74:1269-1274, 1996.

93 Ivan M, Bond JA, Prat M, Comoglio PM, Wynford-Thomas D: Activated ras and ret oncogenes induce over-expression of c-met (hepatocyte growth factor receptor) in human thyroid epithelial cells. Oncogene 14:2417-2423, 1997.

94 Ivan M, Wynford-Thomas D, Jones CJ: Abnormalities of the $P16^{INK4A}$ gene in thyroid cancer cell lines. Eur J Cancer 32A:2369-2370, 1996.

95 Jhiang SM, Caruso D, Gilmore E et al: Detection of the PTC/ret ^{TPC} oncogene in human thyroid cancers. Oncogene 7:1331-1337, 1992.

96 Jhiang SM, Sagartz JE, Tong Q et al: Targeted expression of the Ret/Ptc1 oncogene induces papillary thyroid carcinomas. Endocrinology 137:375-378, 1996.

97 Jiang WG: E-cadherin and its associated protein catenins, cancer invasion and metastasis: Br J Surg 83:437-446, 1996.

98 Jones CJ, Shaw JJ, Wyllie FS et al: High frequency detection of the tumour suppressor gene p16INK4a (MTS1) in human thyroid cancer cell lines. Mol Cell endocrinol 116:115-119, 1996.

99 Jossart G, Epstein H, Shaver J: Immunocytochemical detection of p53 in human thyroid carcinomas is associated with mutation and immortalization of cell lines. J Clin Endocrinol Metab 81:3498-3504, 1996.

100 Kambe F, Seo H: Mediation of the hormone-and serum-dependent regulation of thyroglobulin gene expression by thyroid-transcription factors in rat thyroid FRTL-5 cells. J Endocrinol 150:287-298, 1996.

101 Kameyama K: Expression of MMP-1 in the capsule of thyroid cancer-relationship with invasiveness. Pathol Res Pract 192:20-26, 1996.

102 Karga H, Lee JK, Vickery Al Jr et al: Ras oncogene mutations in benign and malignant thyroid neoplasms. J Clin Endocr Metab 73:832-836, 1991.

103 Katoh R, Bray C, Suzuki K, et al: Growth activity in hyperplastic and neoplastic human thyroid determined by an immunohistochemical staining procedure using monoclonal antibody MIB-1. Hum Pathol 26:139-146, 1995.

104 Klein RD, Sherman D, Ho W-H, et al: A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor. Nature 387:717-721, 1997.

105 Klugbauer S, Lengfelder E, Demidchik EP, Rabes HM: A new form of RET rearrangement in thyroid carcinomas of children after the Chernobyl reactor accident. Oncogene 13:1099-1102, 1996.

106 Knudson AG, Strong LC, Anderson DE: Heredity and cancer in man. In Progress in medical genetics, vol 9, AG Steinberg and AG Bearns, Eds. New York, Grune & Stratton., 1973.

107 Komminoth P: The RET proto-oncogene in medullary and papillary thyroid carcinoma. Molecular features, pathophysiology and clinical implications. Virchows Arch 431:1-9, 1997.

108 Komorowski RA, Hanson GA: Occult thyroid pathology in the young adult: An autopsy study of 138 patients without clinical thyroid disease. Hum Pathol 19:689-696, 1988.

109 Kulig E, Lloyd RV: Transcription factors and endocrine diseases. Endoc Pathol 7:245-250, 1996.

110 Lanzi C, Borrello MG, Bongarzone I, et al: Identification of the product of two oncogenic rearranged forms of the RET proto-oncogene in papillary thyroid carcinomas. Oncogene 7:2189-2194, 1992.

111 Laycock JF and Wise PH. Essential Endocrinology, 3rd edition. Oxford University Press, Oxford, 1996.

112 Lazzereschi D, Ranieri A, Mincione G et al: Human malignant thyroid tumors displayed reduced levels of transforming growth factor beta receptor type II messenger RNA and protein. Cancer Res 57:2071-2076, 1997.

113 Leivo LJ, Frassila K, Virtanen I: Changes in the distribution of integrins and their basement membrane ligands during development of human thyroid follicular epithelium. Histochem J 29:337-345, 1997.

114 Lemoine NR, Hughes CM, Gullick WJ, Brown C L, Wynford-Thomas D: Abnormalities of the epidermal growth factor receptor system in human thyroid neoplasia. Int J Cancer 49: 558- 561 1991.

115 Lemoine NR, Mayall ES, Wyllie FS et al: Activated ras oncogene in human thyroid cancers. Cancer Res 48:4459-4463, 1988.

116 Lemoine NR, Mayall ES, Wyllie FS et al: High frequency of ras oncogene activation in all stages of human thyroid tumorigenesis. Oncogene 4:159-164, 1989.

117 Lemoine NR, Staddon S, Bond J et al: Partial transformation of human thyroid epithelial cells by mutant Ha-ras oncogene. Oncogene 5:1833-1837, 1990

118 Lemoine NR, Wyllie FS, Lillehaug JR et al: Absence of abnormalities of the c-erbB-1 and c-erbB-2 proto-oncogenes in human thyroid neoplasia. Eur J Cancer 26: 777-779, 1990.

119 Le Roithe D, Werner H, Beitner-Johnson D, Roberts CT: Molecular and cellular aspects of the insulin-like growth factor I receptor. Endocrin Rev 16:143-163, 1995.

120 Levine AJ: p53, the cellular gatekeeper for growth and division. Cell 88: 323-331, 1997

121 Li Volsi VA. Surgical pathology of the thyroid. Philadelphia: WB Saunders Company, 1990.

122 Limbert E: Factores prognósticos nos carcinomas diferenciados da tireoideia de origem folicular. PhD Thesis (in portuguese), Lisboa, 1990.

123 Lloyd RV: Growth factors. Endoc Pathol 8:121-127, 1997.

124 Lloyd RV, Jin L, Qian X, Kulig E: Aberrant p27 KIP1 expression in endocrine and other tumors. Am J Pathol 150:401-407, 1997

125 Lyons J, Landis CA, Harsh G et al: Two G proteins oncogenes in human endocrine tumors. Science 249:655-659, 1990.

126 Maciel RMB, Kimura ER, Takahashi MH, et al: Insulin-like growth factor I in human thyroid tissue: Specific localization by immunohistochemistry and *in situ* hybridization. Endocr Pathol 6:207-215, 1995.

127 Manenti G, Pilotti S, Re FC, Della Porta G, Pierotti MA: Selective activaction of *ras* oncogenes in follicular and undifferentiated thyroid carcinomas. Eur J Cancer 30A: 987-993, 1994.

128 Manetto V, Lorenzini R, Cordon-Cardo C, et al: Bcl-2 and Bax expression in thyroid tumours. An immunohistochemical and western blot analysis. Virchows Arch 430:125-130, 1997.

129 Mars WM, Kim T-H, Stolz DB, Liu M-L, Michalopoulous K: Presence of urokinase in serum-free primary rat hepatocyte cultures and its role in activating hepatocyte growth factor. Cancer Res 56:2837-2843, 1996.

130 Matias-Guiu X, Cuatrecasas M, Musulen E, Prat J: p53 expression in anaplastic carcinoma arising from thyroid papillary carcinomas. J Clin Pathol 47:337-339, 1994.

131 Mazzaferri E, Jhiang S: Long-term impact of initial surgery and medical therapy of papillary and follicular thyroid cancer. Am J Med 97:418-427, 1994.

132 McCornick D, Hall PA: The complexities of proliferating cell nuclear antigen. Histopathology 21;591-594, 1992.

133 Miozzo M, Pierotti MA, Sozzi G et al: Human TRK proto-oncogene maps to chromosome 1q32-q34. Oncogene 5:1411-1414, 1990.

134 Mizukami Y, Nonomura A, Hashimoto T et al: Immunohistochemical demonstration of epidermal growth factor and c-myc oncogene product in normal, benign and malignant thyroid tissues. Histopathology 18:11-18, 1991.

135 Mizukami Y, Nonomura A, Hashimoto T et al: Immunohistochemical demonstration of ras p21 oncogene product in normal, benign and malignant human thyroid tissues. Cancer 61: 873-880, 1988.

136 Nakamura T, Yana I, Kobayashi T et al: p53 gene mutations associated with anaplastic transformation of human thyroid carcinomas. Jpn J Cancer Res 83:1293-1298, 1992.

137 Namba H, Gutman RA, Matsuo K, Alvarez A, Fagin JA: H-ras protooncogene mutations in thyroid neoplasms. J Clin Endocr Metab 71:223-229, 1990.

138 Namba H, Rubin SA, Fagin JA: Point mutations of ras oncogenes are an early event in thyroid tumorigenesis. Mol Endocr 4:1474-1479, 1990.

139 Namba H, Yamashita S, Pei HC et al: Lack of PTC gene (ret proto-oncogene rearrangement) in human thyroid tumors. Endocr Jpn 38:627-632, 1991.

140 Ness GO, Haugen DR, Varhaug JE, Akslen LA, Lillehaug JR: Cytoplasmic localization of EGF receptor in papillary thyroid carcinomas: association with the 150-Kda receptor form. Int J Cancer 65:161-167, 1996.

141 Nikiforov Y, Gnepp DR, Fagin J: Thyroid lesions in children and adolescents after the Chernobyl disaster: Implications for the study of radiation tumorigenesis. J Clin Endocrinol Metab 81: 9-14, 1996.

142 Nikiforov Y, Gnepp DR: Pediatric thyroid cancer after the Chernobyl disaster. Cancer 74: 748-766, 1994.

143 Nikiforov Y, Nikiforova M, Gnepp DR, Fagin J: Prevalence of mutations of ras and p53 in benign and malignant thyroid tumors from children exposed to radiation after the Chernobyl nuclear accident. Oncogene 13:687-693, 1996.

144 Nilsson M: Actions of epidermal growth factor and its receptor in the thyroid. Trends Endocrinol Metab 6:175-182, 1995.

145 Nishida T, Nakao K, Hamaji M, Nakahara M, Tsujimoto M: Overexpression of p53 protein and DNA content are important biologic prognostic factors for thyroid cancer. Surgery 119: 568-575, 1996.

146 O'Sullivan C, Barton CM, Staddom SL, Brown CL, Lemoine NR: Activating point mutations of the gsp oncogene in human thyroid adenomas. Mol Carcinog 4:345-349, 1991.

147 Ortiz L, Zannini M, Lauro R, Santisteban P: Transcriptional control of the forkhead thyroid transcription factor TTF-2 by thyrotropin, insulin and insulin-like growth factor I. J Biol Chem 12:23334-23339, 1997.

148 Papadimitriou K, Yiagnisis M, Tolis G, Spandidos DA: Immunohistochemical analysis of the ras oncogene protein product in human thyroid neoplasms. Anticancer Res 8:1223-1227, 1988.

149 Pelosi G, Zamboni G: Proliferation markers and their uses in the study of endocrine tumors. Endocr Patol 7:103-119, 1996.

150 Perucho M: Microsatellite instability: the mutator that mutates the other mutator. Nature Med 2:630-631, 1996.

151 Pilotti S, Collini P, Rilke F et al: Bcl-2 protein expression in carcinomas originating from the follicular epithelium of the thyroid gland. J Pathol 172:337-342, 1994.

152 Poblete MT, Nualart F, del Pozo M et al: Alpha1-antitrypsin expression in human thyroid papillary carcinoma. Am J Surg Pathol 20:956-963, 1996.

153 Pollina L, Pacini F, Fontanini G, et al: bcl-2, p53 and proliferating cell nuclear antigen expression is related to the degree of differentiation in thyroid carcinomas. Br J Cancer 73: 139-143, 1996.

154 Prat M, Narsimhan RP, Crepaldi T, et al: The receptor encoded by the human *c-Met* oncogene is expressed in hepatocytes, epithelial cells and solid tumours. Int J Cancer 49:323-328, 1991.

155 Pujol P, Daures J-P, Nsakala N et al : Degree of thyrotropin suppression as a prognostic determinant in differentiated thyroid cancer. J Clin Endocrinol Metab 81: 4318-4323, 1996.

156 Riese II DJ, Kim ED, Elenius K et al: The epidermal growth factor receptor couples transforming growth Factor-α, heparin-binding epidermal growth factor-like factor, and amphiregulin to Neu, ErbB-3, and ErbB-4. J Biol Chemist 271:20047-20052, 1996.

157 Rigaud C, Bogomoletz WV: Apparent lack of usefulness of monoclonal antibody Ki-67 in thyroid tumour pathology.Relation to histological typing and classification. Pathol Res Pract 187:198-200, 1991.

158 Rossi DL, Acebron A, Santisteban P: Function of the homeo and paired domain proteins TTF-1 and Pax-8 in thyroid cell proliferation. J Chem Biol 29:23139-23142, 1995.

159 Ruco LP, Ranalli T, Marzullo A et al: Expression of *Met* protein in thyroid tumours. J Pathol 180:266-270, 1996.

160 Russo D, Arturi F, Schlumberger M, Caillou B et al: Activating mutations of the TSH receptor in differentiated thyroid carcinomas. Oncogene 11:1907-1911, 1995.

161 Russo D, Arturi F, Wicker R, et al: Genetic alterations in thyroid hyperfunctioning adenomas. J Clin Endocrinol Metab 80:1347-1351, 1995.

162 Russo D, Tumino S, Arturi F et al: Detection of an activating mutation of the thyrotropin receptor in a case of an autonomously hyperfunctioning thyroid insular carcinoma. J Clin Endocrinol Metab 82:735-738, 1997.

163 Ruter A, Dreifus J, Jones M, Nishiyama R, Lennquist S: Overexpression of p53 in tall cell variants of papillary thyroid carcinoma. Surgery 120:1046-1050, 1996.

164 Sagartz JE, Jhiang SM, Tong Q, Capen CC: Thyroid-stimulating hormone promotes growth of thyroid carcinomas in transgenic mice with targeted expression of the re/PTC1 oncogene. Lab Invest 76:307-318, 1997.

165 Salvatore D, Celetti A, Fabien N et al: Low frequency of p53 mutations in human thyroid tumours: p53 and ras mutation in two out of fifty-six thyroid tumours. Eur J Endocrinol 134:177-183, 1996.

166 Sampson RJ, Key CR, Buncher CR, Iijima S: Smallest forms of papillary carcinoma of the thyroid. A study of 141 microcarcinomas less than 0.1 cm in greatest dimension. Arch Pathol 91:334-339, 1971.

167 Santoro M, Carlomagno F, Hay ID et al: Ret oncogene activation in human thyroid neoplasms is restricted to the papillary subtype. J Clin Invest 89:1517-1522, 1992.

168 Santoro M, Sabino N, Ishizaka Y et al: Involvement of ret oncogene in human tumours: specificity of ret activation to thyroid tumours. Br J Cancer 68:460-464, 1993.

169 Santoro M, Tallini G, Carlomagno F et al: Activaction of the RET proto-oncogene in human thyroid tumors. J. Endocrinol. Invest 19; (Suppl. to n°6), 4, 1996.

170 Sapi Z, Lukacs G, Sztan M, Olah E: Contribution of p53 gene alterations to development of metastatic forms of follicular thyroid carcinoma. Diagn Mol Pathol 4:256-260, 1995.

171 Scheumman GF, Hoang-Vu C, Cetin Y et al: Clinical significance of E-cadherin as a prognostic marker in thyroid carcinomas. J Clin Endocrinol Metab 80:2168-2172, 1995.

172 Serini G, Trusolino L, Saggiorato E et al: Changes in integrin and E-cadherin expression in neoplastic *versus* normal thyroid tissue. J Natl Cancer Inst 88:442-449, 1996.

173 Seruca R, Santos NR, David L, et al: Sporadic gastric carcinomas with microsatellite instability display a particular clinicopathologic profile. Int J Cancer 64:32-36, 1995.

174 Shi Y, Zou M, Farid NR, Al-Sedairy ST: Evidence of gene deletion of p21(WAF1/CIP1), a cyclin-dependent protein kinase inhibitor, in thyroid carcinomas. Br J Cancer 74:1336-1341, 1996.

175 Shi Y, Zou M, Schmidt H, Juasz F, Stensky V, Robb D, Farid NR: High rates of codon 61 mutation in thyroid tumors in an iodide-deficient area. Cancer Res 51:2690-2693, 1991.

176 Shimizu T, Usuda N, Yamanda T, Sugenoya A, Iida F: Proliferative activity of human thyroid tumors evaluated by proliferating cell nuclear antigen/cyclin immunohistochemical studies. Cancer 71:2807-2812, 1993.

177 Shiozaki H, Kadowaki T, Doki Y et al: Effect of epidermal growth factor on cadherin-mediated adhesion in a human oesophageal cancer cell line. Br J Cancer 71:250-258, 1995.

178 Shiozaki H, Oka H, Inoue M, Tamura S, Monden M: E-cadherin mediated adhesion system in cancer cells. Cancer 77:1605-1613, 1996.

179 Simon D, Goretzki PE, Schafer C et al: Molecular analysis of mutations in thyroid tumors with TGGE. Exp Clin Endocrinol Diabetes 103:275-279, 1995.

180 Simon D, Goretzki PE, Schafer C et al: Significance of p53 in human thyroid tumors. World J Surg 18:535-540, 1994.

181 Smith P, Wynford-Thomas D, Stringer B, Williams ED: Growth factor control of rat thyroid follicular cell proliferation. Endocrinology 119:1439-1445, 1986.

182 Sobrinho-Simões M, Sambade MC, Gonçalves V: Latent thyroid carcinoma at autopsy. A study from Oporto, Portugal. Cancer 43:1702-1706, 1979.

183 Song B-P: Immunohistochemical demonstration of epidermal growth factor receptor and ceruloplasmina in thyroid diseases. Acta Pathol Jpn 41:336-343, 1991.

184 Sozzi G, Bongarzone I, Miozzo M et al: Cytogenetic and molecular genetic characterization of papillary thyroid carcinomas. Genes Chromos Cancer 5:212-218, 1992.

185 Spambalg D, Sharifi N, Elisei R et al: Structural studies of the thyrotropin receptor and Gsα in human thyroid cancers: low prevalence of mutations predicts infrequent involvement in malignant transformation. J Clin Endocrinol Metab 81:3898-3901, 1996.

186 Suarez HG, du Villard JA, Caillou B et al: gsp mutations in human thyroid tumours. Oncogene 6:677-679, 1991.

187 Suarez HG, du Villard JA, Severino M et al: Presence of ras oncogene activation in all three ras genes in human thyroid tumors. Oncogene 5:565-570, 1990.

188 Takahashi MH, Thomas GA, Williams ED: Evidence for mutual interdependence of epithelium and stromal lymphoid cells in a subset of papillary carcinomas. Br J Cancer 72:813-817, 1995.

189 Tanaka T, Umeki K, Yamamoto I et al: Immunohistochemical loss of thyroid peroxidase in papillary thyroid carcinoma: strong suppression of peroxidase gene expression. J Pathol 179:89-94, 1996.

190 Tannapfel A, Yasui W, Yokozaki H, Wittekind C, Tahara E. Effect of hepatocyte growth factor on the expression of E- and P-cadherin in gastric carcinoma cell lines. Virchows Archiv 425:139-144, 1994.

191 Thomas GA, Davies HG, Williams ED: Site of production of IGF1 in the normal and stimulated mouse thyroid. J Pathol 173:355-360, 1994.

192 Tode B, Serio M, Rotella CM, et al: Insulin-like growth factor-I: autocrine secretion by human thyroid follicular cells in primary culture. J Clin Endocrinol Metab 69:639-647, 1989.

193 Tong Q, Li Y, Smanik PA et al: Characterization of the promoter region and oligomerization domain of H4(D10S170), a gene frequently rearranged with the *ret* proto-oncogene. Oncogene 10:1781-1787, 1995.

194 Trupp M, Arenas E, Fainzilber M et al: Functional receptor for GDNF encoded by the c-ret proto-oncogene. Nature 381:785-789, 1996.

195 Tsarfaty I, Rong S, Resau JH et al: The met proto-oncogene mesenchymal to epithelial cell conversion. Science 263:98-101, 1994.

196 Tung WS, Shevlin DW, Bartsch D, et al: Infrequent CDKN2 mutation in human differentiated thyroid cancers. Mol Carcinog 15:5-10, 1996.

197 Umeki K, Tanaka T, Yamamoto I et al: Differential expression of dipeptidyl peptidase IV (CD26) and thyroid peroxidase in neoplastic thyroid tissues. Endocr J 43:53-60, 1996.

198 van Biesen T, Luttrell LM, Hawes BE, Lefkowitz J: Mitogenic signaling via G protein-coupled receptors. Endocr Rev 17:698-714, 1996.

199 van Heyningen V: One gene-four syndromes. Nature 367:319-320, 1994.

200 Vassart G, van Sande J, Tonacchera M et al: Activating mutations of the TSH receptor gene cause thyroid diseases. Ann endocrinol 57:50-54, 1996.

201 Viale G, Dell'Orto P, Coggi G, Gambacorta M: Coexpression of cytokeratins and vimentin in normal and diseased thyroid glands. Lack of diagnostic utility of vimentin immunostaining. Am J Surg Pathol 13:1034-1040, 1989.

202 Viglietto G, Chiappetta G, Martinez-Tello F et al: RET/PTC oncogene activaction is an early event in thyroid carcinogenesis. Oncogene 11: 1207-1210, 1995.

203 Vleminckx K, Vakaet, Jr. L, Mareel M, Fiers W, Van Roy F: Genetic manipulation of E-cadherin expression by epithelial tumour cells reveals an invasion suppressor role. Cell 66: 107-119, 1991.

204 Vogelstein B, Fearon ER, Hamilton SR, et al: Genetic alterations during colorectal-tumor development. N Eng J Med 319:525-532, 1988.

205 von Wasielewski R, Rhein A, Werner M et al: Immunohistochemical detection of E-cadherin in differentiated thyroid carcinomas correlates with clinical outcome. Cancer Res 57:2501-2507, 1997.

206 Wajjwalku W, Nakamura S, Hasegawa Y et al: Low frequency of rearrangements of the ret and TRK proto-oncogenes in japanese thyroid papillary carcinomas. Jpn J Cancer Res 83:671-675, 1992.

207 Waldmann V, Rabes HM: Absence of Gsα gene mutations in childhood thyroid tumors after Chernobyl in contrast to sporadic adult thyroid neoplasia. Cancer Res 57:2358-2361, 1997.

208 Wallin G, Backdahl M, Christensson B, Grimelius L, Auer G: Nuclear protein content and Ki-67 immunoreactivity in nonneoplastic and neoplastic thyroid cells. Anal Quant Cytol Histol 14:296-303, 1992.

209 Westermark K, Lundqvist M, Wallin G et al: EGF-receptors in human normal and pathological thyroid tissue. Histopathology 28:221-227, 1996.

210 Williams DW, Williams ED, Wynford-Thomas D: Evidence for autocrine production of IGF-IR in human thyroid adenomas. Mol Cell Endocrinol 61:139-143, 1989.

211 Williams DW, Williams ED, Wynford-Thomas D: Loss of dependence on IGF-1 for proliferation of human thyroid adenoma cells. Br J Cancer 57:535-539, 1988.

212 Williams DW, Wynford-Thomas D, Willians ED: Control of human thyroid follicular cell proliferation in suspension and monolayer culture. Mol Cell Endocrinol 51:33-40, 1987.

213 Williams ED: Pathology and molecular biology of post Chernobyl thyroid carcinoma. J Endocrinol Invest 19 (Suppl. to n°6), 4, 1996.

214 Williams GH, Rooney S, Thomas GA, Cummins G, Williams ED: Ret activation in adult and childhood papillary thyroid carcinoma using a reverse transpcriptase-n-polymerase chain reaction approach on archival-nested material. Br J Cancer 74:585-589, 1996.

215 Williams GH, Thomas GA, Voscoboinik L et al: Rearrangements of the RET oncogene in papillary carcinoma and gender. J Endocrinol Invest19 (Suppl. to n°6), 6, 1996.

216 Woolner LB, Lemmon ML, Bearhs OH, Black BM, Keating FR: Occult papillary carcinoma of the thyroid gland- a study of 140 cases observed in a 30-year period. J Clin Endocrinol Metab 20:89-105, 1960.

217 Wrana J, Pawson T: Mad about SMADs. Nature 388:28-29, 1997.

218 Wright P, Lemoine NR, Goretzki PE et al: Mutation of the p53 in a differentiated human thyroid carcinoma cell line, but not in primary thyroid tumors. Oncogene 6:1693-1697, 1991.

219 Wright PA, Lemoine NR, Mayall ES et al: Papillary and follicular thyroid carcinomas show a different pattern of ras oncogene mutation. Br J Cancer 60:576-577, 1989.

220 Wu R-Y, Durick K, Songyang Z et al: Specificity of LIM domain interactions with receptor tyrosine kinases. J Biol Chem 271:15934-15941, 1996.

221 Wyllie FS, Dawson T, Bond JA: Correlated abnormalities of transforming growth factor β1 response and p53 expression in thyroid epithelial cell transformation. Mol Cell Endocr 76:13-21, 1991.

222 Wyllie FS, Haughton MF, Blaydes JP, Schlumberger M, Wynford-Thomas D: Evasion of p53-mediated growth control occurs by three alternative mechanisms in transformed thyroid epithelial cells. Oncogene 10:45-59, 1995.

223 Wyllie FS, Lemoine NR, Williams E, Wynford-Thomas D: Structure and expression of nuclear oncogenes in multi stage thyroid tumorigenesis. Br J Cancer 60:561-565, 1989.

224 Wynford-Thomas D: Molecular genetics of thyroid cancer. Trends Endocrinol Metab 4:224-232, 1993.

225 Wynford-Thomas D: Proliferative lifespan checkpoints: cell-type specificity and influence on tumour biology. Eur J Cancer 33:716-726, 1997.

226 Yamamoto Y, Izumi K, Otsuka H: An immunohistochemical study of epithelial membrane antigen, cytokeratin and vimentin in papillary thyroid carcinoma. Recognition of lethal and favorable prognostic types. Cancer 70:2326-2333, 1992.

227 Yoshida A, Kamma H, Asagsa T, Masuzawa C, Kawahara S, Mimura T, Ito K: Proliferative activity in thyroid tumors. Cancer 69:2548-2552, 1992.

228 Yoshimoto K, Iwahana H, Fukuda A et al: ras mutations in endocrine tumors: mutation detection by polymerase chain reaction-single strand conformation polymorphism. Jpn J Cancer Res 83:1057-1062, 1992.

229 Yoshimoto K, Iwahana H, Fukuda A, Sano T, Saito S, Itakura M: Role of p53 mutations in endocrine tumorigenesis: mutation detection by polymerase chain reaction - single strand conformation polymorphism. Cancer Res 52:5061-5064, 1992.

230 Zanetti A, Stoppacciaro A, Marzullo A, Ruco LP: Expression of c-met-protein, urokinase-type plasminogen activator receptor (uPA-R), and E-cadherin/catenin complex in papillary carcinoma of the thyroid. Pathol Res Pract 193:327 ,1997 (Abstract).

231 Zanini M, Francis-Lang H, Plachov D, Di Laurp R: Pax-8, a paired domain-containing protein, binds to a sequence overlapping the recognition site of a homeodomain and activates transcription from two thyroid-specific promoters. Mol Cell Biology 12:4230-4241, 1992.

232 Zeki K, Spambalg D, Sharifi N, Gonsky R, Fagin JA: Mutations of the adenomatous polyposis coli gene in sporadic thyroid neoplasms. J Clin Endocrinol Metab 79:1317-1321, 1994.

SUMÁRIO E CONCLUSÕES

Realizámos uma série de trabalhos sobre genes e/ou proteínas eventualmente relacionados com o desenvolvimento do carcinoma papilar da tireoide com o objectivo de procurar contribuir para a compreensão de algumas características clinico-patológicas particulares desta neoplasia (crescimento lento, metastização ganglionar muito frequente, bom prognóstico).

1. Proliferative activity of human thyroid tumours evaluated by proliferating cell nuclear antigen/cyclin. Immunohistochemical studies - Estudámos em 76 lesões da tireoide o índice proliferativo. A análise foi realizada por imunocitoquímica utilizando um anticorpo anti-PCNA (posteriormente estudámos também, com resultados semelhantes, o índice proliferativo usando o anticorpo anti-MIB-1 e a citometria de fluxo). Detectámos nos 12 carcinomas papilares estudados um índice proliferativo muito baixo, não significativamente diferente dos indices proliferativos das lesões benignas e dos carcinomas foliculares. Verificámos também que os carcinomas papilares apresentam índices proliferativos significativamente mais baixos do que os detectados em carcinomas pouco diferenciados e em carcinomas indiferenciados.

2. Immunohistochemical detection of p53 in differentiated, poorly differentiated and undifferentiated carcinomas of the thyroid. Avaliámos a expressão imunocitoquímica do produto do gene p53 em 79 lesões da tireoide. Não detectámos imunorreactividade em nenhum dos 12 carcinomas papilares estudados, em contraste com o sucedido em carcinomas foliculares (20% de casos positivos), carcinomas pouco diferenciados (16%) e carcinomas indiferenciados (83%).

3. Expression of c-erb B2 in tumours and tumour like-lesions of the thyroid. Estudámos por imunocitoquímica e por "western-blot" a expressão da proteína c-erb B2 em 34 lesões da tireoide, utilizando dois anticorpos que reconhecem epítopes diferentes. Por "southern-blot" procedemos à pesquisa de amplificação do gene. Nove dos 10 carcinomas papilares estudados mostraram imunorreactividade citoplasmática para o c-

erb B2 , similar à encontrada nas outras lesões da tireoide e na tireoide normal. A proteína revelou por "western-blot", tanto nos carcinomas papilares como nas outras lesões, um peso molecular inferior ao atribuído à proteína "madura" do c-erb B2 (130-140 Kda em vez de 185 Kda). Não detectámos amplificação génica em nenhum caso. Ao contrário do referido na literatura, os carcinomas papilares não mostraram quaisquer especificidades relativamente à expressão do c-erb B2.

4. Benign and malignant thyroid lesions show instability at microsatellite loci. Pesquisámos instabilidade de microssatélites em 46 lesões da tireoide utilizando o método de PCR e cinco ou seis loci por caso. Dos 12 carcinomas papilares estudados dois mostraram instabilidade em um ou mais loci: um num locus (1/12 = 8,3%) e outro em três loci (1/12 = 8,3%). Não detectámos em nenhum caso qualquer mutação do gene hMSH2 (gene de reparação de erros de emparelhamento do ADN mais vezes envolvido na instabilidade de microssatélites). Concluímos que a frequência de ocorrência de instabilidade de microssatélites em carcinomas papilares é baixa e semelhante à detectada em outras lesões (benignas e malignas) da tireoide.

5. E-cadherin gene alterations are rare events in thyroid tumors. Avaliámos a expressão da proteína caderina-E por imunocitoquímica em 31 lesões da tireoide. A técnica do PCR/SSCP foi utilizada para a pesquisa de mutações. A expressão da caderina-E nos 18 carcinomas papilares estudados revelou-se de intensidade reduzida e distribuição heterogénea em comparação com as observadas na tireoide normal e em tumores foliculares benignos e malignos. Detectámos uma mutação do gene da caderina-E no único caso de variante esclerosante difusa de carcinoma papilar incluído na série. Nos outros carcinomas papilares detectámos apenas, como noutros tipos de lesões benignas e malignas, alguns polimorfismos do gene. Concluímos que as alterações do gene da caderina-E parecem ocorrer raramente em carcinomas papilares comuns da tireoide e avançámos a hipótese de que a modulação da transcrição deste gene poderá estar na base da expressão anormal desta proteína em carcinomas papilares.

6. Sporadic *ret*-rearranged papillary carcinoma of the thyroid: A subset of slow growing, less aggressive thyroid neoplasms?. Pesquisámos por "southern-blot" rearranjos do gene ret numa série de 33 carcinomas papilares da tireoide. Os oito casos que tinham rearranjo deste gene foram comparados com os 25 casos sem rearranjo relativamente a diversas características clínico-patológicas e à expressão imunocitoquímica das seguintes proteínas: MIB-1, caderina-E, c-erb B2, c-met/HGFR , bcl-2 e vimentina. A idade média dos doentes com carcinomas papilares com rearranjo do ret é significativamente mais baixa do que a dos doentes com carcinomas que não apresentam rearranjo. Não encontrámos diferenças significativas relativamente às outras características clínico-patológicas estudadas nestes dois grupos para além da tendência para os carcinomas com rearranjo do ret apresentarem um padrão papilar francamente dominante. Os carcinomas papilares com rearranjo do ret mostraram redução significativa de marcação citoplasmática para a caderina-E e uma tendência para menores índices proliferativos do que os casos sem rearranjo do ret. Observámos ainda uma correlação positiva entre a marcação imunocitoquímica do receptor tirosina-cinase c-met/HGFR e a marcação para o filamento intermediário vimentina. Nesta mesma série de carcinomas papilares procedemos ao estudo do receptor do activador do plasminogéneo do tipo uroquinase (uPAR); a expressão deste antigénio correlacionou-se significativamente com a presença de invasão venosa. As conclusões destes trabalhos estão resumidos nas respostas ás questões 2 e 4 (ver adiante).

Com base nos resultados obtidos nestes diferentes estudos e na revisão da literatura procurámos responder às seguintes questões:

1. Existirá no carcinoma papilar da tireoide alguma alteração fundamental da regulação da proliferação e/ou da estabilidade genómica?

Como questão suplementar interrogámo-nos sobre a possibilidade de existir nestes carcinomas uma progressão do tipo "mutadora" (associada a alterações de enzimas de reparação de erros de emparelhamento do ADN) ou do tipo "supressora" (associada a inactivação de genes supressores tumorais).

Concluímos que o carcinoma papilar da tireoide é uma neoplasia pouco proliferativa, não se distinguindo a este respeito das lesões benignas da tireoide. Tão-pouco se distingue no que diz respeito à chamada "instabilidade genómica" uma vez que a instabilidade de microssatélites se revelou um fenómeno restrito nos carcinomas papilares, como aliás nas restantes lesões tireoideias. Concluímos, por isso, que a progressão do carcinoma papilar não parece envolver um mecanismo do tipo "mutador" com alterações de enzimas de reparação de erros de emparelhamento do ADN. O fenótipo dos carcinomas indiferenciados da tireoide (mutações do gene p53, aneuploidia e alterações cromossómicas) sugere, por outro lado, que a progressão de alguns (muitos?) carcinomas papilares para formas indiferenciadas se fará por inactivação de genes supressores tumorais.

2. Estarão receptores de factores de crescimento especificamente desregulados no carcinoma papilar da tireoide?

Para a resposta a esta questão, que é amplamente debatida em trabalhos dos grupos de Akslen, Williams e Wynford-Thomas, somente adicionámos alguns dados no contexto da expressão de receptores tirosina-cinase em carcinomas papilares. Verificámos que a

expressão da proteína do c-erb B2 não se encontra associada ao carcinoma papilar, nem em termos absolutos (é tambem expressa em outras lesões e na tireoide normal) nem relativos. Verificámos também a inexistência de amplificação do gene e a existência de uma proteína com um peso molecular inferior ao do esperado (proteína não glicosilada?). Concluímos assim, em contraste com o "dogma" reinante, que o c-erb B2 e a sua expressão parecem estar mais associados à diferenciação do que à malignização nas lesões tireoideias em geral e no carcinoma papilar em particular.

Em consonância com o grupo de Ruco verificámos a expressão sistematicamente aumentada da proteína do c-met/HGFR em carcinomas papilares e demonstrámos a associação deste facto à expressão aumentada de vimentina. Tal como com o c-erb B2 não encontrámos, nos carcinomas papilares, alterações estruturais do gene c-met/HGFR. A resposta à questão inicial é, portanto, "sim, há evidência para afirmar a desregulação de vários conjuntos de factores de crescimento/receptores de factores de crescimento no carcinoma papilar da tireoide". E justifica-se acrescentar que essa desregulação parece ser muito mais do foro "funcional" (alterações de transcrição, tradução e/ou pós-tradução) do que do foro "estrutural" se exceptuarmos o domínio de alguns receptores tirosina-cinases (ret e trk).

3. O fenótipo metastático particular do carcinoma papilar está associado a alterações na molécula de adesão caderina-E?

Concluímos que as mutações do gene da caderina-E são raras em carcinomas papilares comuns embora haja expressão diminuída da proteína nestes tumores. Esta expressão diminuída poderá explicar a facilidade com que muitos carcinomas papilares progridem no parênquima tireoideu e invadem linfáticos e até tecidos extratireoideus. As mutações do gene da caderina-E poderão, no entanto, ser importantes em variantes particulares do carcinoma papilar caracterizadas por grande invasibilidade local e regional. Verificámos que a expressão aumentada do receptor para o activador do tipo uroquinase associa-se à presença de invasão venosa nos carcinomas papilares e poderá portanto

contribuir para o entendimento da extrema "invasibilidade" destas neoplasias apesar do seu baixo índice proliferativo. A resposta à questão inicial passa assim pela noção de que, tal como com os conjuntos de factores de crescimento/receptores de factores de crescimento, há evidência suficiente para afirmar o envolvimento de alterações da -caderina-E, como de outras moléculas de adesão (ex. CD44), no comportamento biológico particular do carcinoma papilar; parece-nos também lícito afirmar que esse envolvimento reflecte muito mais uma desregulação a nível transcricional e/ou pós-transcricional do que alterações estruturais dos respectivos genes.

4. Qual o significado da activação do oncogene ret em carcinomas papilares da tireoide?

Verificámos que os carcinomas papilares com rearranjo do ret são carcinomas com baixo índice proliferativo que ocorrem em indivíduos jovens e quase nunca evoluem para formas indiferenciadas, em contraste com os carcinomas papilares sem rearranjo que tendem a ocorrer em doentes mais idosos e são susceptíveis de progredir, usualmente através de mutações no gene p53, para carcinomas indiferenciados. Para além destes aspectos não encontrámos diferenças entre as características clínico-patológicas dos casos com e sem rearranjo do ret. Tão pouco observámos diferenças histológicas e imunocitoquímicas entre estes dois grupos de carcinomas papilares excepto no que se refere à franca predominância de arquitectura papilar e à reduzida expressão citoplasmática da caderina-E nos carcinomas papilares com rearranjo do ret. Concluímos haver evidência suficiente para sugerir que a activação do ret produz uma espécie de curto-circuito no processo de carcinogénese papilar levando ao aparecimento, em doentes jovens, de carcinomas papilares bem "desenvolvidos" embora frequentemente de pequenas dimensões; estes carcinomas não progridem aparentemente para formas indiferenciadas, podendo progredir ou mantendo indefinidamente o aspecto de (pequenos) carcinomas de arquitectura papilar, constituindo assim uma espécie de fenómeno "bonsai" a traduzir uma via alternativa de cancerização da tireoide.

PAPER I

Correspondence

Proliferative Activity of Human Thyroid Tumors Evaluated by Proliferating Cell Nuclear Antigen/Cyclin Immunohistochemical Studies

We read with interest the article by Shimizu et al.[1] on the immunohistochemical study of the proliferative activity of human thyroid tumors evaluated by proliferating cell nuclear antigen (PCNA) in which the authors demonstrate the usefulness of this method of assessing proliferation in spite of the reported "complexities of proliferating cell nuclear antigen".[2] Two of the conclusions of the article deserve, in our opinion, a commentary.

The first conclusion concerns the relationship between the ratio of PCNA-positive nuclei and the clinical behavior of thyroid tumors. We concur with Shimizu et al.[1] that clinically aggressive thyroid carcinomas have a higher percentage of PCNA-positive nuclei than do less aggressive tumors. This conclusion is particularly obvious with regard to undifferentiated (anaplastic) carcinomas and, in our hands, poorly differentiated (insular) carcinomas as described by Carcangiu et al.[3] Using the monoclonal antibody PC10 (DAKO) and counting at least 1000 nuclei per section, we found that poorly differentiated carcinomas displayed a significantly higher percentage ($P < 0.05$) of PCNA-positive cells (11.5 ± 2.2%) than did benign lesions (3.6 ± 1.9%), papillary carcinomas (4.4 ± 1.5%), and follicular carcinomas (2.9 ± 1.2%). The same held true for undifferentiated carcinomas, which displayed a significantly higher percentage ($P < 0.01$) of PCNA-positive cells (38.7 ± 3.9%) than did poorly differentiated carcinomas (11.5 ± 2.2%).

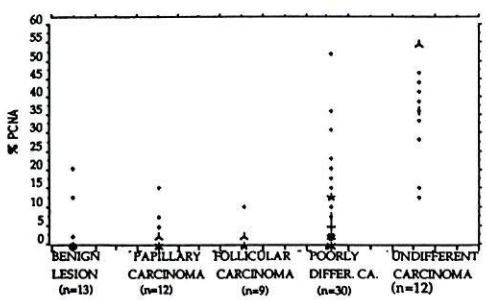


Figure 1. Scattergram of the percentages of immunoreactive cells for proliferating cell nuclear antigen in the different groups of thyroid lesions (three cases were excluded because of equivocal staining for PCA).

Our results are almost identical to those reported by Shimizu et al.,[1] despite the different antibodies used in the two series. In contrast to Shimizu et al.,[1] however, we did not find any significant difference between benign lesions (adenomas and adenomatous goiters) and well differentiated (papillary and follicular) carcinomas. As a matter of fact, some of the benign lesions of our series had higher PCNA-labeling indexes than the maximum score observed in papillary and follicular carcinomas (Fig. 1). Our results correspond with the reports of several groups on the same issue using both flow cytometry[4,5] and Ki-67 immunohistochemistry,[6] thus ruling out the usefulness of the evaluation of cell proliferation by PCNA immunohistochemistry (or by any other method) in the separation of benign from malignant thyroid tumors in single, difficult cases.

The second conclusion of the article of Shimizu et al.[1] we would like to address is the similarity of the results obtained in their study of the so-called well differentiated and poorly differentiated types of papillary carcinomas (4.9 ± 1.7% and 4.6 ± 1.1%, respectively). These results correspond with our own findings using flow cytometry (data not shown) and contribute to raise doubts on the clinico-pathologic meaning of the aforementioned classification of papillary thyroid carcinomas, as has already been stressed by Carcangiu et al.[7] and Vickery et al.[8]

References

1. Shimizu T, Usuda N, Yamanda T, Sujenoya A, Iida F. Proliferative activity of human thyroid tumors evaluated by proliferative cell nuclear antigen/cyclin immunhistochemical studies. *Cancer* 1993; 71:2807–12.
2. McCormick D, Hall PA. The complexities of proliferating cell nuclear antigen. *Histopathology* 1992; 21:591–4.
3. Carcangiu ML, Zampi G, Rosai J. Poorly differentiated ("insular") thyroid carcinoma: a reinterpretation of Langhans' "wuchernde Struma". *Am J Surg Pathol* 1984; 8:655–68.
4. Johannessen J, Sobrinho-Simões M, Lindmo T, Tangen K. The diagnostic value of flow cytometry DNA measurements in selected disorders of the human thyroid. *Am J Clin Pathol* 1982; 77: 20–5.
5. Hostetter A, Hrafnkelsson J, Wingren S, Enestrom S, Nordenskjold B. A comparative study of DNA cytometry methods for benign and malignant thyroid tissue. *Am J Clin Pathol* 1988; 89: 760–3.
6. Rigaud C, Bogomoletz W. Apparent lack of usefulness of monoclonal antibody Ki-67 in thyroid tumour pathology: relation to histological typing and classification. *Pathol Res Pract* 1991; 187: 198–200.
7. Carcangiu ML, Zampi G, Pupi A, Castagnoly A, Rosai J. Papilary carcinoma of the thyroid: a clinicopathologic study of 241 cases

treated at the University of Florence, Italy. *Cancer* 1985; 55:805–28.

8. Vickery AL Jr., Carcangiu ML, Johannessen JV, Sobrinho-Simões M. Papillary carcinoma. *Semin Diagn Pathol* 1985; 2:90–100.

Paula Soares, M. Sc., Ph.D.
Manuel Sobrinho-Simões, M.D.
Institute of Molecular Pathology and Immunology
Medical Faculty of the University of Porto
Hospital S. João
4200 Porto
Portugal

PAPER II

Int. J. Cancer: **56,** 459–461 (1994)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

LETTER TO THE EDITOR

Dear Sir,

Expression of C-*erb* B2 in tumours and tumour-like lesions of the thyroid

Immunoreactivity for C-erb B2 protein, frequently owing to C-erb *B2 gene amplification, occurs in some human carcinomas of the breast, salivary gland, stomach, colon, pancreas and bladder (Slamon* et al., *1987; De Potter* et al., *1989; Kameda* et al., *1990). In breast carcinomas in particular, the amplification and over-expression of* C-erb*B2 gene has been shown to correlate with biological aggressiveness of the neoplasms, therefore being considered a useful prognostic indicator (Slamon* et al., *1987). C-erb B2 protein expression has also been detected in normal mammary epithelial cells and has been shown to depend on cell density and levels of epidermal growth factor (EGF) in the culture medium, thus pointing to a role of* C-erb *B2 gene in normal mammary gland physiology (Kornilova* et al., *1992). Thyroid gland growth and differentiation result from the balance between the primary tissue-specific regulator TSH and "secondary" regulators such as EGF and insulin-like growth factor-1 (Smith and Wynford-Thomas, 1989). The role played by these and other growth factors and oncogenes in the development and maintenance of thyroid tumours is still a matter of intense debate (Low, 1991). The results obtained to date on the expression of EGFR and* C-erb *B2 gene in the pathogenesis of thyroid lesions are particularly controversial. Aasland* et al. *(1988) reported higher levels of C-erb B2 mRNA in papillary carcinomas than in other thyroid lesions and suggested the existence of an association of the EGFR/C-erb B2 product with thyroid carcinoma. These findings contradict those of Lemoine* et al. *(1990) who did not find any immunohistochemical expression and/or DNA abnormalities of C-erb B2 in normal thyroid or in thyroid tumours. Haugen* et al. *(1992) reported the detection of C-erb B2 immunoreactivity in 12 out 17 papillary carcinomas, while no C-erb B2 protein immunostaining was seen in any other type of thyroid tumour or in non-neoplastic thyroid tissue.*

These controversies prompted us to study the C-erb *B2 gene and C-erb B2 protein in a series of different tumours and tumour-like lesions of the thyroid and in normal thyroid tissue.*

The expression of C-erb B2 protein was evaluated by immunohistochemistry on paraffin sections of 34 thyroid lesions classified according to Hedinger et al. *(1988) as nodular goitre (n = 4), adenoma (n = 6), follicular carcinoma (n = 4), papillary carcinoma (n = 10), poorly differentiated (insular) carcinoma (n = 6), medullary carcinoma (n = 3) and undifferentiated carcinoma (n = 1) and in the normal adjacent thyroid parenchyma of 25 of the 34 cases. Immunostaining was performed through the avidin-biotin-peroxidase complex as previously described (Holm* et al., *1985). Two monoclonal antibodies were used (OM-11-952, Cambridge Research Biochemicals, diluted 1:500, and NCL-CB11, Novocastra (Newcastle upon Tyne, UK), diluted 1:40). Positive controls included a case of gastric carcinoma previously known to have intense cellular membrane immunoreactivity and 8-fold amplification of* C-erb *B2 (David* et al., *1992) and the gastric cancer cell line MKN45 which is known to over-express* C-erb *B2 without gene amplification (Kameda* et al., *1990). Negative controls consisted of substitution of the primary antibody for mouse immunoglobulins of the same subclass and in the same concentration as the primary antibody. The results were semi-quantitatively scored as negative (−), few positive cells (+), 10 to 50% positive cells (++), and more than 50% positive cells (+++) by 2 observers acting independently. The same procedure was used to score the mean staining intensity of positive cells into faint and intense. Statistical comparison of the results was performed using the Chi-square method after Yate's correction and Fisher's exact test. Six cases (and the respective normal thyroid parenchyma from 2 of them) were studied by Western-blot analysis. Total proteins were extracted and separated by electrophoresis in polyacrylamide/SDS gels (5%) running at 20mA for 2 hr with molecular weight standards (RPN756, Amersham, Aylesbury, UK) and then blotted onto a nitrocellulose filter (Hybond-C, Amersham) for 2 hour at 400mA in a buffer containing 25mM TRIS, 19 mM glycine, pH8.3. Non-specific binding was inhibited by 1 hr incubation in a blocking buffer (5% dry milk, 0.1% Tween − 20 in TBS). The blot was probed with OM-11-952 (diluted 1:1,000) and NCL-CB11 (diluted 1:40) and colour development was performed using a streptavidin-biotin-alkaline phosphatase system (RPN 22, Amersham) following the manufacturer's recommendations. To evaluate gene amplification, high-molecular-weight DNA was extracted according to a standard procedure (Mullenbach* et al., *1989) from 8 tumours scored ++ or +++ by immunohistochemistry. DNA samples were digested with Eco RI, separated by electrophoresis in 0.8% agarose gel and transferred to nylon membranes (Hybond N, Amersham). Hybridization was performed using a* ^{32}*P-labelled* C-erb *B2 cDNA probe (Yamamoto* et al., *1986). The probe was labelled by an oligolabelling method (Feinberg and Vogelstein, 1983) and autoradiograms were developed after 4 days at −70°C.*

The results of the immunohistochemical study are summarized in Table I. Twenty-nine of the 34 cases showed immunoreactivity with both antibodies. Immunoreactivity was also observed in all normal thyroid samples studied. There was a striking variation from case to case in the percentage of positive cells and only a minority of lesions displayed more than 50% of cells positive for the C-erb B2 protein (Table I). This finding fits with the well-known thyroid-cell heterogenity regarding both growth and/or functional status (Miyamoto et al., *1988; Zbieranowski and Murray, 1992). The pattern of staining was almost identical with both antibodies, the difference being a trend towards more intense staining and slightly more numerous immunoreactive cells with OM-11-952 than with NCL-CB11 antibody. Every positive case showed cytoplasmic staining and in several thyroid lesions the staining was localized in the supranuclear region of the cytoplasm (Figs. 1, 2). No distinct cell-membrane staining was observed in thyroid lesions or in normal thyroid samples (Figs. 1, 2). Colloid was non-*

TABLE I – SUMMARY OF THE IMMUNOHISTOCHEMICAL FINDINGS

Diagnosis (n)	Staining score				Staining intensity	
	0 n (%)	+ n (%)	++ n (%)	+++ n (%)	Faint n (%)	Intense n (%)
Goitre (4)	0 (0)	0 (0)	2 (50)	2 (50)	0 (0)	4 (100)
Adenoma (6)	0 (0)	4 (66)	1 (17)	1 (17)	3 (50)	3 (50)
Follicular carcinoma (4)	0 (0)	2 (50)	1 (25)	1 (25)	3 (75)	1 (25)
Papillary carcinoma (10)	1 (10)	4 (40)	4 (40)	1 (10)	6 (60)	3 (30)
Poorly differ. carcinoma (6)	2 (33)	1 (17)	2 (33)	1 (17)	1 (11)	3 (50)
Undifferentiated carcinoma (1)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)
Medullary carcinoma (3)	2 (66)	0 (0)	1 (33)	0 (0)	0 (0)	1 (33)
Normal thyroid (25)	1 (4)	12 (48)	7 (28)	5 (20)	9 (36)	16 (64)

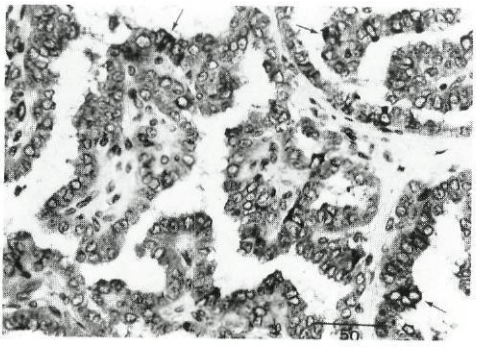


FIGURE 1 – Papillary carcinoma showing few immunoreactive cells for C-erb B2 protein (arrows). The tumour was scored +(NCL-CB11 antibody) (scale bar = 50 mm).

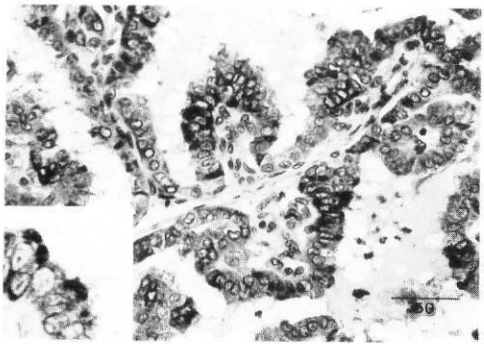


FIGURE 2 – Papillary carcinoma with relatively abundant immunoreactive cells for C-erb B2 protein. The tumour was scored ++. *Inset:* Higher magnification showing the supranuclear localization of the (intense) staining (NCL-CB11 antibody) (scale bar = 50 mm; the magnification of the inset is twice as great). The Western blot showed a band of 130–140 kDa. Gene amplification was not found in this case by Southern blot.

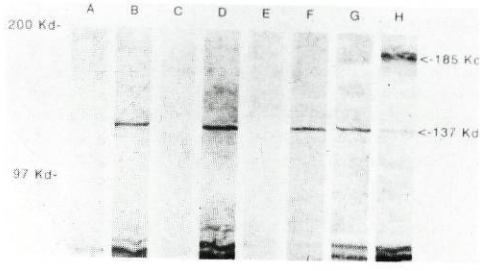


FIGURE 3 – Western-blot analysis of 5 lesions and 2 adjacent normal thyroid tissue specimens. Lanes A and B, normal thyroids scored respectively as + and +++; lane C, nodular goitre scored as ++; lanes D and E, follicular adenomas scored respectively as +++ and +; lane F, papillary carcinoma scored as +++; lane G, follicular carcinoma scored as +++; lane H, gastric carcinoma (positive control). The typical band of 185 kDa detected in the positive control was not seen in any thyroid sample. A band of 133–137 kDa was observed in every lesion scored +++ as well as in the positive control. The absence of a distinct 133–137-kDa band in the goitre (lane C) probably reflects the bias induced by cell heterogeneity of most lesions. Low-molecular-weight bands were seen in every sample. (See text for details.)

immunoreactive. No significant differences were found between the different types of thyroid lesion. In the Western-blot analysis, a band of 130–140 kDa was identified with both antibodies (Fig. 3). Other bands of molecular weight lower than 130 kDa were observed with both antibodies in every immunohistochemically positive sample, though more prominently with OM-11-952 than with NCL-CB11. Western blots yielded a good correlation between immunohistochemical staining and the density of the bands (Fig. 3; results not shown in detail). The typical 180–190-kDa band was only clearly observed in the gastric carcinoma tissue and the gastric cancer cell line used as positive controls. Gene amplification was not found in any of the 8 cases studied, in contrast to the gastric carcinoma used as positive control (results not shown).

It is impossible to compare our results with those reported so far on the immunohistochemical detection of C-erb B2 protein in thyroid lesions (Lemoine et al., *1990; Haugen* et al., *1992) as different antisera were used. The reported studies did not include OM-11-952 or NCL-CB11 among the antibodies tested and this may explain the differing results of the 3 series. It is noteworthy that, in our preliminary tests to determine the working conditions, we included the polyclonal antibody OA-11-854 used by Haugen* et al. *(1992) on frozen material. This antibody was withdrawn from the present study because, in our hands, consistent results in paraffin-embedded material were not obtained.*

Using 2 antibodies considered as specific for C-erb B2 protein, we observed immunoreactivity in all types of thyroid

lesion and in normal thyroid parenchyma as well. To a certain extent our results agree with the findings of Haugen et al. *(1992) concerning the detection of C-erb B2 mRNA in all cases included in their series. Taking these results together, it seems likely that C-erb B2 protein may serve as a growth factor involved in the growth and differentiation control in thyroid as, apparently, in the mammary gland (Kornilova* et al., *1992). The absence of C-*erb *B2 amplification reported by Lemoine* et al. *(1990) and confirmed in the present study also agrees with the aforementioned hypothesis.*

Our results raise an additional problem: the detection by Western blot of a 130–140-kDa band instead of the typical 180–190-kDa band. The meaning of this finding is still not clear. Since we confirmed our first results with a second, different monoclonal antibody, we think the possibility of this being just a spurious finding may be excluded. As suggested by Corbett et al. *(1990) in the light of their own results, we may be identifying a precursor molecule of the C-*erb *B2 gene product rather than its usual, final form. Relating the molecular weight of the detected band (130–140 kDa) with the molecular weight (about 137 kDa) of the apoprotein of the C-erb B2 glycoprotein (Akyama* et al., *1986), it is tempting to suggest that one may be dealing with an unglycosylated form of the C-erb B2 protein. This hypothesis fits with the cytoplasmic (Golgi? RER?) localization of the staining we observed in our series, since it is known that deficiently glycosylated glycoproteins tend to stay in the cytoplasmic compartments where they are synthesized instead of proceeding to their cell sites (Daneker* et al., *1987). It is, however, hardly conceivable that only a precursor form can be identified in such a wide range of lesions and even in normal parenchyma. The cytoplasmic staining may also reflect a rapid turnover of the C-erb B2 receptor in the thyroid following activation by its ligands and/or under partial control by EGF, thus leading to its internalization and degradation as suggested in the mammary gland by Kornilova* et al. *(1992). The possibility of testing any of these hypotheses, which are not mutually exclusive, rests beyond the scope of the present study.*

Yours sincerely,

Paula SOARES, *Clara* SAMBADE *and Manuel* SOBRINHO-SIMÕES

Institute of Molecular Pathology and Immunology of the University of Porto-IPATIMUP, 4200 Porto, Portugal.

September 7, 1993.

REFERENCES

AASLAND, R., LILLEHAUG, J.R., MALE, R., JOSENDAL, O., VARHAUG, J.E. and KLEPPE, K., Expression of oncogenes in thyroid tumours: co-expression of *c-erbB-2/neu* and *c-erbB*. *Brit. J. Cancer,* **57,** 358–363 (1988).

AKYAMA, T., SUDO, C., OGAWARA, H., TOYOSHIMA, K. and YAMAMOTO, T., The product of the human C-*erbB2* gene: a 185 kilodalton glycoprotein with tyrosine kinase activity. *Science,* **123,** 1644–1646 (1986).

CORBETT, I.P., HENRY, J.A., ANGUS, B., WATCHORN, C.J., WILKINSON, L., HENNESSY, C., GULLICK, W.J., TUZI, N.L., MAY, F.E.B., WESTLEY, B.R. and HORNE, C.H.W., NCL-CB11, a new monoclonal antibody recognizing the internal domain of the C-*erbB*-2 oncogene protein effective for use on formalin-fixed, paraffin-embedded tissue. *J. Pathol.,* **161,** 15–25 (1990).

DANEKER, G., MERCURIO, A., GUERRA, L., WOLF, B., SALEM, R., BAGLI, D. and STEELE, G., Laminin expression in colorectal carcinomas varying in degree of differentiation. *Arch. Surg.,* **122,** 1470–1474 (1987).

DAVID, L., SERUCA, R., NESLAND, J.M., SOARES, P., SANSONNETY, F., HOLM, R., BORRESEN, A.-L. and SOBRINHO-SIMÕES, M., C-*erb* B2 expression in primary gastric carcinomas and their metastases. *Mod. Pathol.,* **5,** 384–390 (1992).

DE POTTER, C.R., VAN DAELE, S., VAN DE VIJVER, M.J., PAUWELS, C., MAERTENS, G., DE BOEVER, J., VANDEKERCKHOVE, D. and ROELS, H., The expression of the *neu* oncogene product in breast lesions and in normal fetal and adult human tissues. *Histopathology,* **15,** 351–362 (1989).

FEINBERG, A.P. and VOGELSTEIN, B., A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.,* **132,** 6–13 (1983).

HAUGEN, D.F.R., AKSLEN, L.A., VARHAUG, J.E. and LILLEHAUG, J.R., Expression of C-erbB-2 protein in papillary thyroid carcinomas. *Brit. J. Cancer,* **65,** 832–837 (1992).

HEDINGER, C., WILLIAMS, ED. and SOBIN, L.H., *Histologic typing of thyroid tumours. World Health Organization—International Histological Classification of Tumours.* Springer, Berlin (1988).

HOLM, R., SOBRINHO-SIMÕES, M., NESLAND, J.M., GOULD, V.E. and JOHANNESSEN, V., Medullary carcinoma of the thyroid gland. An immunohistochemical study. *Ultrastruct. Pathol.,* **8,** 25–41 (1985).

KAMEDA, T., YASUI, W., YOSHIDA, K., TSUJINO, T., NAKAYAMA, H., ITO, M., ITO, H. and TAHARA, E., Expression of *ERB B2* in human gastric carcinomas: Relationship between p185 *ERB B2* expression and the gene amplification. *Cancer Res.,* **50,** 8002–8009 (1990).

KORNILOVA, T., TAVERNA, D., HOECK, W. and HYNES, N.E., Surface expression of erb B2 protein is post-transcriptionally regulated in mammary epithelial cells by epidermal growth factor and by the culture density. *Oncogene,* **7,** 511–519 (1992).

LEMOINE, N.R., WYLLIE, F.S., LILLEHAUG, J.R., STADDON, S.L., HUGHES, C.M., AASLAND, R., SHAW, J., VARHAUG, J.E., BROWN, C.L., GULLICK, W.J. and WYNFORD-THOMAS, D., Absence of abnormalities of the c-*erbB*-1 and c-*erbB*-2 proto oncogenes in human thyroid neoplasia. *Europ. J. Cancer,* **26,** 777–779 (1990).

LOW, M., Oncogenes and thyroid tumors: scratching the surface. *Endocr. Pathol.,* **2,** 120–122 (1991).

MIYAMOTO, M., SUGAWA, H., MORI, T., HASE, K., KUMA, K. and IMURA, H., Epidermal growth factor receptors on cultured neoplastic human thyroid cells and effects of epidermal growth factor and thyroid-stimulating hormone on their growth. *Cancer Res.,* **48,** 3652–3656 (1988).

MULLENBACH, R., WELDAR, C. and LAGODA, P., An efficient salt chloroform extraction of DNA from blood and tissues. *Trends Genet.,* **5,** 391 (1989).

SLAMON, D.J., CLARK, G.M., WONG, S.G., LEVIN, W.J., ULLRICH, A. and MCGUIRE, W.L., Human breast cancer: correlation of relapse and survival with amplification of the *HER-2/neu* oncogene. *Science,* **235,** 177–182 (1987).

SMITH, P. and WYNFORD-THOMAS, D., Control of thyroid follicular cell proliferation—cellular aspects. *In:* D. Wynford-Thomas and E.D. Williams (eds.), *Pathobiology of thyroid tumours,* pp. 67–89, Churchill Livingstone, London (1989).

YAMAMOTO, T., IKAWA, S., AKIYAMA, T., SEMBA, K., NOMURA, N., MIYAMA, N., SAITO, T. and TOYOSHIMA, K., Similarity of protein encoded by the human *c-erbB*-2 gene to epidermal growth factor receptor. *Nature (Lond.),* **319,** 230–234 (1986).

ZBIERANOWSKI, I. and MURRAY, D., The study of endocrine tumors by flow and image cytometry. *Endocr. Pathol.,* **3,** 63–82 (1992).

PAPER III

Histopathology 1994, **24**, 205–210

Immunohistochemical detection of *p53* in differentiated, poorly differentiated and undifferentiated carcinomas of the thyroid

P.SOARES, J.CAMESELLE-TEIJEIRO* & M.SOBRINHO-SIMÕES

*Institute of Molecular Pathology and Immunology—IPATIMUP, Medical School of the University of Porto, Porto, Portugal and *Department of Pathology, General Hospital of Galicia, Santiago, Spain*

Date of submission 29 March 1993
Accepted for publication 22 September 1993

SOARES P., CAMESELLE-TEIJEIRO J. & SOBRINHO-SIMÕES M.
(1994) *Histopathology* **24**, 205–210

Immunohistochemical detection of *p53* in differentiated, poorly differentiated and undifferentiated carcinomas of the thyroid

In an attempt to find whether or not *p53* immunoreactivity in the thyroid gland is restricted to undifferentiated carcinomas and to evaluate the putative prognostic usefulness of its detection, we investigated *p53* immunoreactivity in a series of 14 benign thyroid lesions and 65 thyroid carcinomas (12 papillary; six minimally invasive follicular; four widely invasive follicular; 31 poorly differentiated and 12 undifferentiated tumours). Unequivocal nuclear immunostaining for *p53* was observed in two widely invasive follicular carcinoma (20.0%), five poorly differentiated carcinomas (16.1%) and in 10 undifferentiated carcinomas (83.3%). The percentage of immunoreactive cells was much smaller in the former groups than in undifferentiated carcinomas. Despite a trend to a more aggressive behaviour of the *p53* immunoreactive cases no significant differences in the outcome of patients with positive and negative tumours was found when the comparison was made within each category of carcinomas. We conclude that *p53* immunoreactivity can be detected both in undifferentiated carcinomas and in some differentiated and poorly differentiated thyroid carcinomas. Larger series of cases are necessary to evaluate the prognostic usefulness of this finding.

Keywords: *p53*, immunohistochemistry, carcinoma, thyroid

Introduction

Mutations of the *p53* tumour suppressor gene play a major role in the development of many carcinomas, namely in the colon, breast and bladder[1–3], whereas the role played by such mutations in thyroid carcinogenesis remains controversial[4–12]. Some authors claim that *p53* mutations are restricted to undifferentiated carcinomas[6,12] thus supporting the concept that in thyroid the inactivation of the *p53* gene is a (very) late event associated with the most advanced stages of tumour progression[6,7,10,12]. In contrast to this, other groups reported the presence of *p53* mutations and/or immunoreactivity in a number of differentiated thyroid carcinomas[5,9,11]. Dobashi *et al.*[9] advanced, furthermore, the possibility of using the over-expression of *p53* as a prognostic factor in human thyroid carcinomas.

In an attempt to help settle the aforementioned controversy and to verify whether or not *p53* immunoreactivity may serve for prognostic purposes, we undertook an immunohistochemical study of a series of thyroid tumours with particular emphasis on the so-called poorly differentiated carcinomas[13–15] which are thought to represent an intermediate step between differentiated and undifferentiated thyroid carcinomas.

Address for correspondence: Professor Manuel Sobrinho-Simões, Department of Pathology, Medical School, Hospital S. João, 4200 Porto, Portugal.

Materials and methods

Fourteen benign thyroid lesions (adenomas and nodular goitres) and 70 thyroid carcinomas from which representative sampling and properly fixed material was

available (one to four paraffin blocks per case) were investigated routinely as well as by immunohistochemistry. In seven carcinomas frozen material was also available for immunohistochemical study. In seven cases two independent lesions could be studied in the same histological sections. In five cases sequential biopsies from the primary tumours and from recurrence or metastases were available for study.

The 70 carcinomas were classified according to the criteria advanced by Hedinger *et al.*[16] and Carcangiu *et al.*[13] as papillary carcinoma ($n=12$), minimally invasive follicular carcinoma (7) widely invasive follicular carcinoma (4), poorly differentiated carcinoma (35) and undifferentiated (anaplastic) carcinoma (12). One minimally invasive follicular carcinoma and four poorly differentiated carcinomas were excluded from the series due to equivocal immunostaining for *p53*. Five follicular carcinomas and one poorly differentiated carcinoma displayed Hürthle cell features. Immunohistochemistry for cytokeratins, thyroglobulin, calcitonin and calcitonin-gene related peptide was used whenever necessary in the differential diagnosis of the most difficult cases.

Paraffin embedded material. The tissues were fixed in formalin or in Bouin fixative, and embedded in paraffin wax. Sections, 5 μm thick, were cut and mounted in gelatin-coated slides. Immunostaining was performed using the avidin–biotin–peroxidase complex (ABC) method. Sections were dewaxed and then immersed for 30 min in 0.3% hydrogen peroxide (H_2O_2) in methanol, to block endogenous peroxidase activity. A proteolytic step was performed subsequently, immersing the slides in 0.1% pepsin, pH 2.5, for 30 min. After several washings in distilled water, non-specific staining was eliminated by 20 min incubation with normal rabbit non-immune serum. Excess normal serum was removed and replaced by the specific primary antibody (Ab6 Oncogene Sciences) diluted 1:75. Sections were incubated overnight at 4°C. After washing the slides, sections were incubated with a 1:100 dilution of biotin-labelled secondary antibody for 30 min and ABC (avidin 1:100 and biotin-labelled peroxidase 1:100, K 355, Dako Corporation) for 1 h. Subsequently sections were stained for 7 min with 0.05% 3,3-diaminobenzidine tetrahydrochloride, 0.01% H_2O_2 in 0.05 M Tris/HCl buffer pH 7.6, counterstained with haematoxylin, dehydrated and mounted. Non-immune serum was diluted in 25% BSA in TBS, and primary antibodies, biotinylated secondary antibodies and ABC were diluted in 12.5% BSA in TBS. Paraffin embedded sections of a gastric carcinoma with a confirmed mutation of *p53* which were unequivocally immunoreactive for *p53*, were included in every series as positive controls. Negative controls consisted in substitution of the primary antibody for mouse immunoglobulin of the same subclass and in the same concentration as the primary antibody.

Frozen material. Immunostaining of the frozen sections was performed with the peroxidase–anti-peroxidase (PAP) method. Briefly, cryostat sections were fixed in acetone for 10 min and hydrated in distilled water. The *p53* antibody (Ab6, Oncogene Sciences) was then added at a concentration of 1:75 and incubated overnight in a moist chamber at 4°C. After careful washes in PBS, a peroxidase conjugated rabbit immunoglobulin to mouse immunoglobulin (Dako, P-260) in a concentration of 1:15, followed by a peroxidase monoclonal mouse anti-peroxidase complex (Dako, P-850) at 1:100, were applied for 30 min each. The staining step was performed as previously described. PBS was used as diluent. Frozen material of the aforementioned gastric carcinoma was used as a positive control for the detection of *p53* immunoreactivity in frozen sections using the PAP method. PBS instead of the Ab6 antibody was used as a negative control.

Immunohistochemical evaluation. Every section was carefully scrutinized for the presence of nuclear immunostaining for *p53*. The areas displaying more numerous stained nuclei were selected for counting. From each case at least 1000 nuclei were counted. *p53* immunoreactivity was expressed as negative (−), less than 10% of positive cells (+), from 10 to 50% of positive cells (++), and more than 50% of positive cells (+++).

Follow-up and statistical analysis. Poorly differentiated carcinomas were treated by total thyroidectomy or quasi-total thyroidectomy followed by radioactive iodine. The treatment of well-differentiated carcinomas and undifferentiated carcinomas varied greatly. Follow-up information was obtained in 36 of the 43 patients with poorly differentiated and undifferentiated carcinomas (83.7%). The results are expressed in percentage or in mean ± standard error. The statistical analysis was performed using the χ^2 method after Yates correction, Fisher's exact test, and Student's two sided *t*-test. The results were considered significantly different if $P<0.05$.

Results

Unequivocal nuclear immunostaining for *p53* was observed in two widely invasive follicular carcinomas (Figure 1), five poorly differentiated carcinomas (Figure 2), and in 10 undifferentiated carcinomas (Figures 3 and 4). The immunohistochemical data are summarized in Table 1.

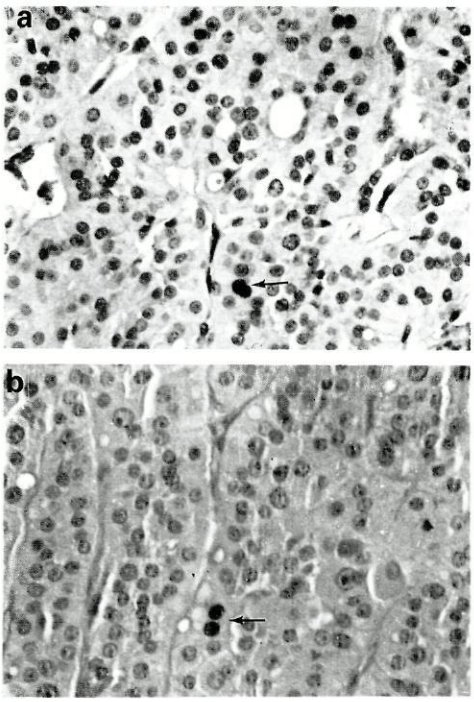


Figure 1. *p53* immunostaining in two different areas of a follicular carcinoma: there are very few positive cells (arrows).

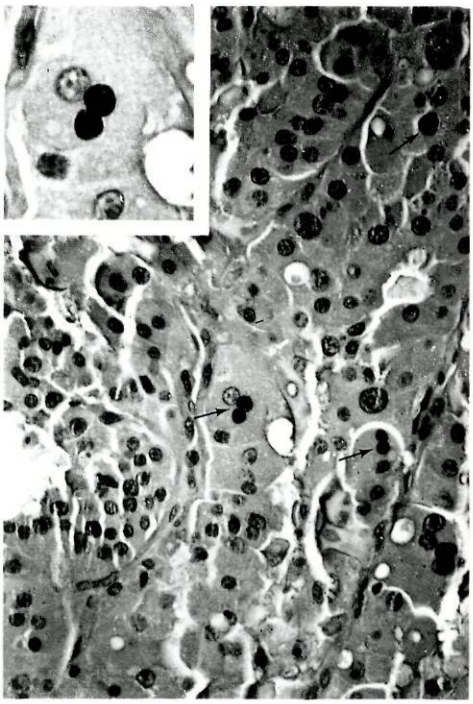


Figure 2. *p53* immunostaining of a poorly differentiated carcinoma displaying Hürthle cells and some positive nuclei (arrows). *Inset:* higher magnification of the cell with two positive nuclei.

Immunostained nuclei were present throughout the neoplastic tissue of all the positive cases but the number of stained nuclei varied greatly for each type of carcinoma. Both follicular carcinomas and four of the five poorly differentiated carcinomas had less than 10% of immunoreactive cells, whereas eight of the 10 positive undifferentiated carcinomas had more than 10% of immunoreactive cells (Table 1). Most of these undifferentiated carcinomas, however, had more than 25% of immunoreactive cells (Figures 3 and 4). In cases with less than 10% of immunoreactive cells, immunostained nuclei tended to be concentrated in some areas that contrasted with large areas completely devoid of immunoreactivity. In one follicular carcinoma only dispersed immunoreactive nuclei were observed (Figure 1).

The staining was more intense when frozen material was used instead of formalin-fixed, paraffin embedded material in two cases that had been classified as positive in paraffin sections. However, none of the five cases of differentiated and poorly differentiated carcinomas that had been considered as negative in paraffin embedded material displayed any detectable staining in the respective frozen samples. Diffuse cytoplasmic immunostaining was observed in these cases belonging to different groups of lesions. A similar pattern of staining was observed in these cases when the antiserum to *p53* had been substituted by mouse immunoglobulin; this staining was thus considered to be non-specific.

The two patients with widely invasive follicular carcinomas positive for *p53* were 72 and 80 years old. One of these patients died 24 months after surgery and the other was lost to follow-up. The series of widely invasive follicular carcinomas was too small to allow any meaningful comparison between positive and negative cases.

The comparison of the clinico-pathological features of the five cases of poorly differentiated carcinoma displaying immunoreactivity for *p53* with those of the 26 negative cases showed a trend to a higher mortality rate at 2 years in the former (75.0%) as compared with the latter (52.4%), without, however, reaching statistical significance. The two undifferentiated carcinomas in

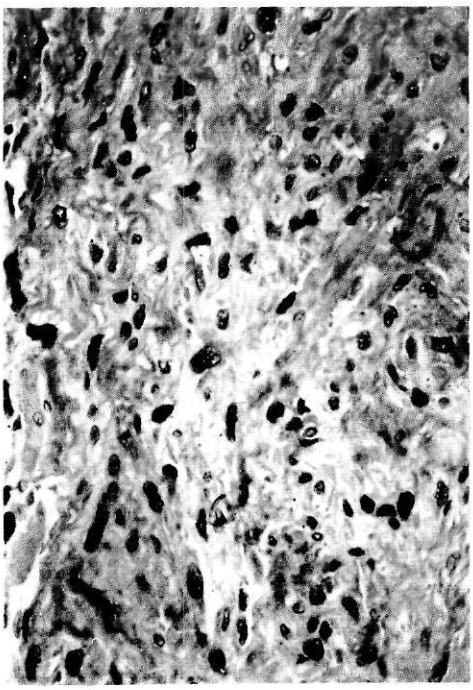


Figure 3. *p53* immunostaining of an undifferentiated carcinoma composed of spindle cells (sarcomatoid appearance) with many ($10\% < n < 50\%$) positive cells.

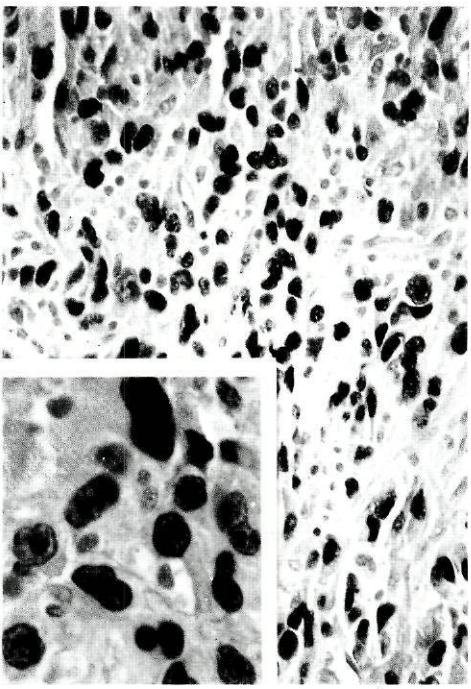


Figure 4. *p53* immunostaining of an undifferentiated carcinoma with many (> 50%) positive cells. *Inset:* higher magnification of some positive cells and some cells which are difficult to classify as positive or negative.

which no immunoreactivity for *p53* was detected did not differ significantly from the 10 positive cases in any respect. Ten of the 11 patients with follow-up were dead within 13 months after surgery. The remaining patient is alive 4 months after surgery.

Comparison of the immunoreactivity for *p53* in two different lesions represented in the same histological sections of seven cases revealed that whenever there was a difference between the two lesions, *p53* immunoreactivity was found in the most malignant one (a poorly differentiated carcinoma close to an adenoma and foci of undifferentiated carcinoma having two poorly differentiated carcinomas as background).

The immunohistochemical study of the five cases in which there were sequential biopsies from recurrences and/or metastases revealed, in three of them, similar *p53* immunoreactivity patterns in the primary and secondary tumours (two negative poorly differentiated carcinomas and one positive undifferentiated carcinoma). One of the remaining cases was a poorly differentiated carcinoma that was positive for *p53* in the primary tumour and displayed equivocal immunostaining in the metastasis, 18 months later. The other case was a poorly differentiated carcinoma which was negative for *p53* and recurred 1 year later as an undifferentiated carcinoma displaying immunoreactivity for *p53*.

Table 1. Summary of the immunohistochemical findings for *p53*

	Staining score			
	0	+	++	+++
Goitre and adenoma (14 cases)	14	0	0	0
Papillary carcinoma (12)	12	0	0	0
Follicular carcinoma (10)	8	2*	0	0
Poorly differentiated carcinoma (31)	26	4	1	0
Undifferentiated carcinoma (12)	2	2	6	2

* These positive follicular carcinomas were of the widely invasive type.

Discussion

To the best of our knowledge this is the first study of *p53* immunoreactivity emphasizing the analysis of so-called poorly differentiated thyroid carcinomas, considered to be an intermediate step between differentiated and undifferentiated carcinomas[13–15]. For practical purposes it is very difficult or even impossible to distinguish this group from the formerly designated 'moderately differentiated follicular carcinomas'[17–18]. Curiously, the existence of some overlap between both classifications is indirectly supported by our results: the *p53* immunoreactive cases of our series that did not belong to the group of undifferentiated carcinomas were either poorly differentiated carcinomas or moderately differentiated, widely invasive follicular carcinomas.

We found *p53* immunoreactivity in the nuclei of 10 out of the 12 (83.3%) undifferentiated carcinomas. This result fits with the high frequency of *p53* mutation(s) reported by Ito *et al.*[6,12] and Fagin *et al.*[11] in the same type of thyroid tumours (Table 2) as it is known that there is a good relationship between the accumulation of *p53* protein to levels detectable by immunohistochemistry and the presence of point mutation(s) of the *p53* gene[19].

The absence of immunoreactivity for *p53* in two undifferentiated carcinomas does not appear to be due to technical artefact (e.g. poor fixation) because the neoplastic cells of these tumours displayed strong immunoreactivity for a number of other antigens. We do not know if these non-immunoreactive cases represent examples of the so-called false negative *p53* immunoreactivity, i.e. cases in which the loss of *p53* activity may not be accompanied by a detectable accumulation of the protein[19]. The similarity of the negative and positive undifferentiated carcinomas regarding the most important clinico-pathological features, including very high PCNA labelling indexes (results not shown), are in keeping with the aforementioned possibility.

The contrast between the prevalence of *p53* mutation and/or immunoreactivity in differentiated and undifferentiated thyroid carcinomas in most of the series supports the assumption that *p53* mutation(s) may be a necessary molecular event in the undifferentiated (anaplastic) transformation of thyroid carcinomas[6]. In keeping with the repeatedly reported absence of *p53* mutations in papillary carcinomas and unlike Dobashi *et al.*[9], we did not find *p53* immunoreactivity in any of the 12 papillary carcinomas of our series. We found, on the other hand, *p53* immunoreactivity in a minority of follicular carcinomas and poorly differentiated thyroid carcinomas. At variance with undifferentiated carcinomas, immunoreactivity for *p53* in these tumours usually involved only a small percentage of neoplastic cells.

Wright *et al.*[5] described the first case of a follicular carcinoma with a *p53* mutation. This tumour was the only positive one out of a series of 129 negative tumours and has given rise to the only available human follicular carcinoma cell line we are aware of[5]. This cell line, which also has the *p53* mutation of the primary tumour, was established only after 132 unsuccessful attempts with other differentiated follicular carcinomas, thus

Table 2. Summary of the results published to date on *p53* expression in thyroid epithelial tumours

	Methods (no. of cases)	Benign lesions	Papillary carcinoma	Follicular carcinoma	Poorly differentiated carcinoma	Undifferentiated carcinoma
Wyllie *et al.*[4]	Southern, Northern blots (36)	15 (0)*	12 (0)	5 (0)	–	4 (0)
Wright *et al.*[5]	Immunohistochemistry, sequencing (129)	57 (0)	28 (0)	24 (4)	–	20 (0)†
Ito *et al.*[6]	PCR, sequencing (16)	–	10 (0)	–	–	6 (83)†
Nakamura *et al.*[7]	PCR–RNAase PA (9)	–	–	–	–	9 (22)
Yoshimoto *et al.*[8]	PCR, SSCP (47)	22 (0)	23 (0)	–	1 (0)	1 (0)†
Dobashi *et al.*[9]	Immunohistochemistry (126)	16 (0)	63 (11)	14 (14)	22 (41)	11 (64)
Donghi *et al.*[10]	Immunohistochemistry, PCR, SSCP (52)	–	33 (0)	4 (0)	8 (25)	7 (70)
Fagin *et al.*[11]	PCR, SSCP (85)	31 (0)	37 (0)	11 (9)	–	6 (83)†
Ito *et al.*[12]	PCR-RFLP, sequencing (21)	–	10 (0)	4 (0)	–	7 (86)†
Present series	Immunohistochemistry (79)	14 (0)	12 (0)	10 (20)	31 (16)	12 (83)

PCR, Polymerase chain reaction; RNAase PA, RNAase protection analysis; SSCP, single strand conformation polymorphism; RFLP, restriction fragment length polymorphism.

* The figures refer to the number of cases. In parentheses is the percentage of positive cases.

† Results of cell lines are excluded.

leading to the conclusion that *p53* mutation(s) probably confers on cells of differentiated thyroid carcinomas the ability to grow in culture[5].

Our finding of *p53* positive nuclei in well-differentiated follicular carcinomas together with the results of Wright *et al.*[5] show that, at least in a number of cases, *p53* mutations may precede the anaplastic transformation in thyroid tumours. If one excludes the undifferentiated carcinomas, the positive cases in our series were restricted to widely invasive follicular carcinomas and poorly differentiated carcinomas, thus reinforcing the concept that mutations of *p53* occur only in the most aggressive groups of thyroid carcinomas[6,10–12]. It remains to be shown if the presence of immunoreactive cells in follicular carcinomas and poorly differentiated carcinomas indicates an increased liability for anaplastic transformation and is therefore a prognostic indicator in routine diagnosis. The number of cases of follicular carcinoma is too small to allow a meaningful comparison of the clinico-pathological features of positive and negative cases. It should be noted, however, that the two positive cases occurred in elderly patients (72 and 80 years) with widely invasive, clinically aggressive follicular carcinomas.

The study of cases with two apparently independent lesions, as well as the study of cases with sequential biopsies from the same patients, did not provide enough evidence to draw a definite conclusion on the prognostic importance of the presence of *p53* immunoreactivity in differentiated and poorly differentiated carcinomas, despite confirming the association between *p53* immunoreactivity and the clinically aggressive forms of thyroid carcinomas. The potential prognostic applications of *p53* immunohistochemistry are hampered by the low prevalence of *p53* immunoreactivity in follicular and poorly differentiated carcinomas. This may change in the future if a more sensitive way of detecting *p53* mutations in routine pathological specimens is found. In undifferentiated carcinomas, the detection of *p53* immunoreactivity appears, from a practical standpoint, to be useless. In fact, our cases displayed similar, very aggressive clinico-pathological features, regardless of the presence or absence of *p53* immunoreactivity.

Acknowledgements

We thank Mrs Dina Leitão for expert technical assistance and Mrs Fátima Magalhães for her help in editing the manuscript. The study was supported by a grant from JNICT (Project PMCT/C/SAU/194/90).

References

1. Silvestrini R, Benini E, Daidone MG. *p53* as an independent prognostic marker in lymph-node negative breast cancer patients. *J. Natl Cancer Inst.* 1993; **85**; 965–970.
2. Baker SJ, Preisinger AC, Jessup JM *et al. p53* gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. *Cancer Res.* 1990; **50**; 7717–7722.
3. Reznikoff CA, Kao C, Messing EM, Newton M, Swaminathan S. A molecular genetic model of human bladder carcinogenesis. *Sem. Cancer Biol.* 1993; **4**; 143–152.
4. Wyllie FS, Lemoine NR, Williams E, Wynford-Thomas D. Structure and expression of nuclear oncogenes in multi stage thyroid tumorigenesis. *Br. J. Cancer* 1989; **60**; 561–565.
5. Wright P, Lemoine NR, Goretzki PE *et al.* Mutation of the *p53* in a differentiated human thyroid carcinoma cell line, but not in primary thyroid tumors. *Oncogene* 1991; **6**; 1693–1697.
6. Ito T, Seyama T, Mizuno T *et al.* Unique association of *p53* mutation with undifferentiated but not differentiated carcinomas of the thyroid gland. *Cancer Res.* 1992; **52**; 1369–1371.
7. Nakamura T, Yana I, Kobayashi T *et al. p53* gene mutations associated with anaplastic transformation of human thyroid carcinomas. *Jpn. J. Cancer Res.* 1992; **83**; 1293–1298.
8. Yoshimoto K, Iwahana H, Fukuda A, Sano T, Saito S, Itakura M. Role of *p53* mutations in endocrine tumorigenesis: mutation detection by polymerase chain reaction—single strand conformation polymorphism. *Cancer Res.* 1992; **52**; 5061–5064.
9. Dobashi Y, Sakamoto A, Sugimura H *et al.* Overexpression of *p53* as a possible prognostic factor in human thyroid carcinoma. *Am. J. Surg. Pathol.* 1993; **17**; 375–381.
10. Donghi R, Longoni A, Pilotti S, Michieli P, Della Porta G, Pierotti MA. Gene *p53* mutations are restricted to poorly differentiated and undifferentiated carcinomas of the thyroid gland. *J. Clin. Invest.* 1993; **91**; 1753–1760.
11. Fagin JA, Matsuo K, Karmakar A, Chen DL, Tang SH, Koeffler HP. High prevalence of mutation of *p53* gene in poorly differentiated human thyroid carcinomas. *J. Clin. Invest.* 1993; **91**; 179–184.
12. Ito T, Seyama T, Mizuno T *et al.* Genetic alterations in thyroid tumor progression: association with *p53* gene mutations. *Jpn. J. Cancer Res.* 1993; **84**; 526–531.
13. Carcangiu ML, Zampi G, Rosai J. Poorly differentiated ('insular') thyroid carcinoma. *Am. J. Surg. Pathol.* 1984; **8**; 655–688.
14. Papotti M, Micca F-B, Favero A, Palestini N, Bussolati G. Poorly differentiated thyroid carcinomas with primordial cell component. A group of aggressive lesions sharing insular, trabecular and solid patterns. *Am. J. Surg. Pathol.* 1993; **17**; 291–301.
15. Sobrinho-Simões M, Sambade C. Poorly differentiated carcinomas of the thyroid gland. A review of the clinico-pathologic, light microscopic, immunohistochemical, electron microscopic, lectin histochemical and morphometric features of a series of 30 cases of this heterogeneous, clinically aggressive group of thyroid tumors. *Prog. Surg. Pathol.* 1994; (in press).
16. Hedinger C, Williams ED, Sobin LH. *Histologic Typing of Thyroid Tumours*. World Health Organization—Histological Classification of Tumours. Berlin: Springer Verlag, 1988.
17. Hedinger C, Sobin LH. *Histologic Typing of Typing Tumours*. International Histological Classification of Tumours no 11. Geneva: World Health Organization, 1974.
18. Harach HR, Franssika KO. Thyroglobulin immunostaining in follicular thyroid carcinoma: relationship to the degree of differentiation and cell type. *Histopathology* 1988; **13**; 43–54.
19. Wynford-Thomas D. *p53* in tumour pathology: can we trust immunocytochemistry? *J. Pathol.* 1992; **166**; 329–330.

PAPER IV

Pergamon

European Journal of Cancer Vol. 33, No. 2, pp. 293–296, 1997

Printed in Great Britain
0959-8049/97 $17.00 + 0.00

PII: S0959-8049(96)00457-1

Original Paper

Benign and Malignant Thyroid Lesions Show Instability at Microsatellite Loci

P. Soares,[1] N.R. dos Santos,[1] R. Seruca,[1] R.A. Lothe[2] and M. Sobrinho-Simões[1]

[1]IPATIMUP, Department of Pathology, Medical Faculty of Porto, Hospital S. João, 4200 Porto, Portugal; and [2]Department Genetics, Institute for Cancer Research, The Norwegian Radium Hospital, 0310 Oslo, Norway

Forty-six benign and malignant tumours and tumour-like lesions of the thyroid were analysed for microsatellite instability (MI) at eight loci, mapping to four different chromosomes, 7 lesions (15%) displayed MI at one or more loci, including 2/13 nodular goitres, 2/15 follicular adenomas, 2/12 papillary carcinomas and 1/4 follicular carcinomas. Two benign and one malignant lesion among the seven unstable cases exhibited this phenotype at three or more loci. We found no mutations in the mismatch repair gene, *hMSH2*, in the seven affected cases, after screening all the exons by CDGE mutation analysis. At variance with the data on record, these results indicate that, despite being relatively infrequent, MI does occur not only in thyroid carcinomas but also in benign lesions (goitres and follicular adenomas of the thyroid).

Key words: thyroid, nodular goitre, follicular adenoma, papillary carcinoma, microsatellite instability

Eur J Cancer, Vol. 33, No. 2, pp. 293–296, 1997

INTRODUCTION

MICROSATELLITE INSTABILITY (MI) is a genetic phenomenon manifested by shifts in the electrophoretic mobility of microsatellite repeat fragments. Mutations (generally small deletions or insertions) occurring during replication remain unrepaired and result in novel alleles. MI was first reported in sporadic and hereditary non-polyposis colorectal carcinoma (HNPCC) [1–3] and thereafter associated with inactivating mutations in the human DNA mismatch repair genes *hMSH2*, *hMLH1*, *hPMS1* and *hPMS2* [4–8]. It was further shown that cells harbouring such mutations were mismatch repair deficient and hypermutable [9, 10].

The prevalence of MI varies greatly in HNPCC-related sporadic carcinomas (colon, stomach, pancreas, ovary and endometrium) [1–3, 11–17], as well as in other types of tumours such as breast, lung and prostate carcinomas [12, 14, 18–22]. There also appears to be large variation in the prevalence of MI in precancerous lesions of different organs [23–26], thus leading to divergent assumptions on the timing of occurrence of this type of genomic instability in tumorigenesis [24, 26, 27].

Correspondence to M. Sobrinho-Simões.
Received 8 May 1996; revised 9 Aug. 1996; accepted 19 Aug. 1996.

A limited number of thyroid carcinomas have been analysed for MI. Nine tumours reported by Vermiglio and associates did not exhibit MI, whereas thyroid carcinomas from 3 patients with multiple primary cancers did show this phenotype [29]. In addition, MI has not been reported, to the best of our knowledge, in any other benign or malignant endocrine tumour. In order to evaluate further this phenomenon in the thyroid, 46 lesions were analysed for instability at eight microsatellite loci.

MATERIALS AND METHODS

Tumour samples (n = 46)

In each case, fresh tissue samples from thyroid nodules were immediately snap frozen in liquid nitrogen and stored at −70°C until use. When available, DNA from normal parenchyma adjacent to the lesions ($n = 18$) was used as a constitutional control, otherwise, we used corresponding peripheral blood DNA ($n = 28$). High-molecular-weight DNA was isolated using standard methods [30]. Classification of the lesions was made according to Hedinger and associates [31] and Rosai and associates [32] into nodular goitre ($n = 13$), follicular adenoma ($n = 15$), papillary carcinoma ($n = 12$), follicular carcinoma ($n = 4$) and poorly differentiated carcinoma ($n = 2$). There was no

Table 1. *Thyroid lesions with microsatellite instability*

Cases	Lesion	Microsatellite markers							Number*
		D1S196	D1S103	D5S346	D2S102	CTLA4	D5S82	D16S265	
1	NG	–	–	–	–	–	MI	–	1/8
2	NG	MI	–	–	MI	–	MI	–	3/8
3	FA	MI	MI	MI	MI	–	–	–	4/8
4	FA	–	–	–	–	–	MI	–	1/8
5	PC	–	–	–	–	–	MI	–	1/8
6	PC	MI	MI	MI	ND	MI	MI	MI	6/7
7	FC	–	–	–	–	–	MI	–	1/8

*Loci with microsatellite instability/loci analysed.
NG, nodular goitre; FA, follicular adenoma; PC, papillary carcinoma; FC, follicular carcinoma; MI, microsatellite instability; ND, not determined; –, normal homozygote or heterozygote and unchanged in tumours.

history of familial cancer syndromes or multiple primary cancers in any of the 46 patients.

Microsatellite marker analysis

Paired samples of tumour and normal control DNA were amplified by PCR (polymerase chain reaction) at the following eight loci containing dinucleotide repeat sequences (chromosomal location in parentheses): D1S103 (1q), D1S196 (1q), D2S102 (2q), CTLA4 (2q), D5S346 (5q), D5S82 (5q), D16S265 (16q) and D16S301 (16q). PCR was performed in 25 μl volumes of a mixture containing 10 mM Tris-HCl (pH 8.0), 50 mM of KCl, 1.5 mM $MgCl_2$, 100 μM of each deoxynucleotide triphosphate except dCTP, 10 μM of dCTP, 1 μCi of [α-^{32}P]dCTP, 0.4 μM of each primer, 0.75 units of *Taq* DNA polymerase, and 30–50 ng of genomic DNA. Thirty cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.75 min were performed, with an initial denaturation step of 94°C for 5 min and a final extension step of 72°C for 10 min using a Perkin-Elmer 9600 GeneAmp PCR System. PCR reaction products were diluted 1:1 with a loading buffer (98% formamide, 0.1% xylene cyanol FF, 0.1% bromophenol blue, and 10 mM EDTA [pH 8.0]) and then denatured for 5 min at 95°C. Subsequently, 2 μl of this solution were electrophoresed in 6% polyacrylamide gels containing 7 M urea and 32.5% formamide for 2–3 h at 60 W. After electrophoresis, gels were fixed in 10% acetic acid, washed, dried and exposed to X-ray field for 12–24 h. All scorings were made independently by two observers. According to Thibodeau and associates [3], genetic alterations involving the gain or loss of two or more repeat units are classified as Type I and alterations of one repeat unit as Type II. Eight loci were analysed per case and, for the sake of simplicity, a tumour was considered positive (MI) when at least one locus displayed a different mobility band. This option does not mean we belittle the existence of aetiopathogenic differences between cases with single-repeat slippage at just one locus and cases with slippage at several loci. Such differences are taken into account in the comparison of the different groups (see Results and Table 1). All RER$^+$ loci were confirmed by a new PCR and electrophoretic run.

hMSH2 *mutation analysis*

All 16 exons of *hMSH2* were screened for mutations using constant denaturant gel electrophoresis (CDGE) [33]. The separation principle of CDGE is based on the unique melting behaviour of each DNA fragment. The detailed procedure for such analysis of *hMSH2* has been recently published [34]. Briefly, theoretical melting profiles were calculated for all exons, followed by primer design using MacMelt 1.0 (MedProbe A/S) and OLIGO (National Biosciences) computer programs. Template DNAs were amplified by PCR, and the products separated by CDGE (D-GENE system, BioRad). If a mutation is indicated by CDGE, sequencing of the affected exon will identify the exact position of the changed base(s). By using the conditions described by Børresen and associates [34], it was estimated that approximately 65% of all mutations will be detected within the analysed fragments.

RESULTS

The results are summarised in Tables 1 and 2. Seven of the 46 thyroid lesions displayed MI at one or more loci (Table 1). Among these, there were two nodular goitres and two follicular adenomas, thus leading to an overall MI prevalence of 14.3% in the 28 benign lesions (Table 2). Two papillary carcinomas and a follicular carcinoma displayed MI, thus leading to a MI prevalence of 16.7% in the 18 carcinomas.

Three out of the seven lesions displayed MI at three or more loci (a nodular goitre (Figure 1)), a follicular adenoma and a papillary carcinoma (Figure 2), whereas the remaining four lesions showed MI at a single locus (Table 1).

Cases 1, 4 and 7, with MI at a single locus, displayed Type II alterations, whereas case 5, also with one altered locus, displayed a Type I alteration (see Materials and Methods). Cases 2 and 3, with three and four unstable loci respectively, displayed both Type I and Type II alterations. Case 6 displayed Type I alterations at all affected loci.

Both papillary carcinomas with MI displayed discrete to moderate lymphoid infiltration which could also be observed in most of the papillary carcinomas without MI. Comparison of the histological features of other lesions with MI with those of their non-unstable counterparts did not disclose any notable difference.

Among the seven microsatellite unstable tumours, no aberrantly migrating bands were observed by CDGE of the 16 *hMSH2* exons, and thereby no mutations were indicated.

Table 2. *Frequency of microsatellite instability in thyroid lesions*

Diagnosis (*n*)	MI ⩾ 1 locus	MI ⩾ 3 loci
Nodular goitre (13)	2 (15.4%)	1 (7.6%)
Follicular adenoma (15)	2 (13.3%)	1 (6.6%)
Papillary carcinoma (12)	2 (16.7%)	1 (8.3%)
Follicular carcinoma (4)	1 (25.0%)	0 (0%)
Poorly differentiated carcinoma (2)	0 (0%)	0 (0%)

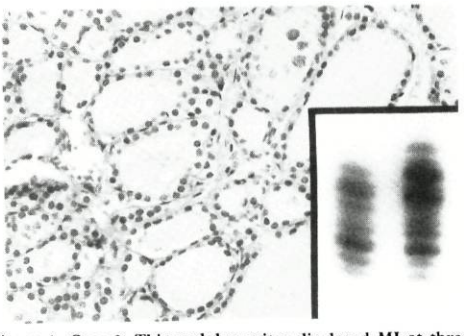


Figure 1. Case 2: This nodular goitre displayed MI at three loci. Haematoxylin and eosin. Inset: PCR amplification of locus D5S82 showing Type II alteration (left lane, normal thyroid; right lane, goitre). Magnification ×125.

Loss of heterozygosity was found at D1S103 and D1S196 in a follicular adenoma and a follicular carcinoma, respectively.

DISCUSSION

Two of the three microsatellite unstable carcinomas were only affected at one out of eight loci, implying that these two cases could have been scored as negative if fewer loci had been analysed. Therefore, the absence of MI in thyroid carcinomas reported by Vermiglio and associates [28] probably reflects the limited number of analysed markers and the small size of their series.

The three thyroid carcinomas with MI reported by Horii and associates [29] were observed in a series of patients with multiple primary cancers, and are thereby not directly comparable to those of our series. The tumours of the present study were from patients with no history of familial cancer nor evidence of other primary cancers. Taking our results together with those of Vermiglio and associates [28], one may conclude that the prevalence of MI in thyroid carcinomas, outside the context of multiple primary cancers, lies below the values reported for HNPCC-related sporadic carcinomas [1–3, 11, 12, 14, 16, 17].

The discrepant prevalences of MI in thyroid carcinomas, prostatic carcinomas [18, 19], and breast carcinomas [20, 21], rules out the possibility of establishing a close relationship between hormone-dependent tumours in general and prevalence of MI.

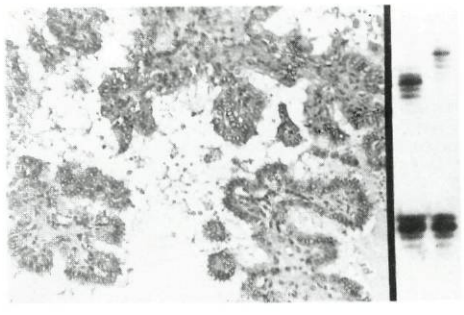


Figure 2. Case 6: This papillary carcinoma displayed MI at six loci. Haematoxylin and eosin. Inset: PCR amplification of locus CTLA4 showing Type I alteration (left lane, normal thyroid; right lane, papillary carcinoma). Magnification ×125.

MI has been detected in lesions of ulcerative colitis, regardless of the presence of dysplasia [35]; Brentnall and associates proposed that MI in this setting could result from saturation of the DNA repair mechanisms due to the stress of chronic inflammation. It is unlikely that such a mechanism is operative in the unstable tumours of the present series, namely in the goitre cases, since none of them displayed signs of inflammation. The occurrence of MI at one or few loci may also represent a random event [36]. This possibility cannot be definitely ruled out especially in those cases exhibiting single locus alterations. The same does not hold true, however, for thyroid lesions with instability at multiple loci—as many as six out of seven unstable loci were observed in one of our cases. We do not know if the use of tri- and tetranucleotide markers would lead to a higher number of altered loci in thyroid tumours, since it is known that different patterns of alterations of short tandem repeats are found in different types of tumours [20, 37–39].

The finding of a subset of benign thyroid tumours and tumour-like lesions displaying MI at several loci suggests that MI can be an early event in thyroid tumorigenesis, thus fitting well with Loeb's hypothesis [40] and with the observation of MI in some precancerous dysplasias of the stomach [24], adenomas and/or dysplasias of the colon [25–27], and Barrett's metaplasia of the oesophagus [23]. Moreover, the occurrence of MI at several loci in a nodular goitre fits with the concept that at least some of the nodules of the so-called adenomatous goitres appear to be neoplastic rather than hyperplastic [41].

We found a similar prevalence of MI in benign lesions and carcinomas of the thyroid, both when the comparison concerns the cases with affected loci, regardless of their number, and when it is restricted to cases with several affected loci, but a larger series is necessary to confirm this finding, which contrasts with the usually much lower frequency of MI in benign lesions than in their malignant counterparts in several organs [23, 24, 26]. In colorectal tumours, the majority of unstable adenomas are affected at a single locus, whereas more than half of the unstable carcinomas show novel alleles at several loci [13, 26], suggesting that these changes accumulate during tumour growth.

We cannot rule out the putative involvement of DNA polymerases at least in the cases presenting alterations at a single locus, since it has been reported that mutations of DNA polymerases may interfere with poly(GT) tract stability [42], whereas the occurrence of MI at multiple loci suggests the existence of abnormalities of DNA mismatch repair genes [42, 43]. Six of seven tumours showed novel alleles at the D5S82 locus, suggesting that this locus is highly unstable.

The mismatch repair gene *hMSH2* is apparently not mutated in any of the microsatellite unstable cases of our series. This may reflect the fact that thyroid carcinoma is not part of HNPCC; the size of the series is, however, too small to allow any definitive conclusion on this issue and it is estimated that only approximately 65% of all mutations of *hMSH2* will be detected within the analysed fragments under the conditions we have used [34]. Additional tumours need to be collected for MI in order to study further *hMSH2* and to search for mutations in other DNA mismatch repair genes to see if we can find in thyroid pathology, as in other systems, a relationship between MI

and abnormalities of at least one mismatch repair gene [34, 44].

1. Aaltonen LA, Peltomäki P, Leach FS, *et al.* Clues to the pathogenesis of familial colorectal cancer. *Science* 1993, **260**, 812–816.
2. Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* 1993, **363**, 558–561.
3. Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. *Science* 1993, **260**, 816–819.
4. Fishel R, Lescoe MK, Rao MRS, *et al.* The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. *Cell* 1993, **75**, 1027–1038.
5. Leach FS, Nicolaides NC, Papadopoulos N, *et al.* Mutation of a mutS homolog in hereditary nonpolyposis colorectal cancer. *Cell* 1994, **75**, 1215–1225.
6. Bronner CE, Baker SM, Morrison PT, *et al.* Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. *Nature* 1994, **368**, 258–261.
7. Papadopoulos N, Nicolaides NC, Wei Y-F, *et al.* Mutation of a mutL homolog in hereditary colon cancer. *Science* 1994, **263**, 1625–1629.
8. Nicolaides NC, Papadopoulos N, Liu B, *et al.* Mutations of two PMS homologues in hereditary nonpolyposis colon cancer. *Nature* 1994, **371**, 75–80.
9. Parsons R, Li G-M, Longley MJ, *et al.* Hypermutability and mismatch repair deficiency in RER$^+$ tumour cells. *Cell* 1993, **75**, 1227–1236.
10. Umar A, Boyer JC, Thomas DC, Nguyen DC, *et al.* Defective mismatch repair in extracts of colorectal and endometrial cancer cell lines exhibiting microsatellite instability. *J Biol Chem* 1994, **269**, 14367–14370.
11. Dos Santos NR, Seruca R, Constância M, Seixas M, Sobrinho-Simões M. Microsatellite instability at multiple loci in gastric carcinoma: clinicopathologic implications and prognosis. *Gastroenterology* 1996, **110**, 38–44.
12. Han H-J, Yanagisawa A, Kato Y, Park J-G, Nakamura Y. Genetic instability in pancreatic cancer and a poorly differentiated type of gastric cancer. *Cancer Res* 1993, **53**, 5087–5089.
13. Lothe RA, Peltomäki P, Meling GI, *et al.* Genomic instability in colorectal cancer: relationship to clinicopathological variables and family history. *Cancer Res* 1993, **53**, 5849–5852.
14. Peltomäki P, Lothe RA, Aaltonen LA, *et al.* Microsatellite instability is associated with tumours that characterize the hereditary non-polyposis colorectal carcinoma syndrome. *Cancer Res* 1993, **53**, 5853–5855.
15. Risinger JI, Berchuck A, Kohler MF, Watson P, Lynch HT, Boyd J. Genetic instability microsatellites in endometrial carcinoma. *Cancer Res* 1993, **53**, 5100–5103.
16. Seruca R, Santos NR, David L, *et al.* Sporadic gastric carcinomas with microsatellite instability display a particular clinicopathologic profile. *Int J Cancer* 1995, **64**, 32–36.
17. Tangir J, Loughridge NS, Berkowitz RS, *et al.* Frequent microsatellite instability in epithelial borderline ovarian tumours. *Cancer Res* 1996, **56**, 2501–2505.
18. Gao X, Wu N, Grignon D, *et al.* High frequency of mutator phenotype in human prostatic adenocarcinoma. *Oncogene* 1994, **9**, 2999–3003.
19. Watanabe M, Imai H, Shiraishi J, Shimazaki J, Kotake T, Yatani R. Microsatellite instability in human prostate cancers. *Br J Cancer* 1995, **72**, 562–564.
20. Wooster R, Cleton-Jansen A-M, Collins N, *et al.* Instability of short tandem repeats (microsatellites) in human cancers. *Nature Genet* 1994, **6**, 152–156.
21. Aldaz CM, Chen T, Sahin A, Cunningham J, Bondy M. Comparative allelotype of *in situ* and invasive human breast cancer: high frequency of microsatellite instability in lobular breast carcinomas. *Cancer Res* 1995, **55**, 3976–3981.
22. Fong KM, Zimmerman PV, Smith PJ. Microsatellite instability and other molecular abnormalities in non-small cell lung cancer. *Cancer Res* 1995, **55**, 28–30.
23. Meltzer SJ, Yin J, Manin B, *et al.* Microsatellite instability occurs frequently and in both diploid and aneuploid cell population of Barrett's associated esophageal adenocarcinoma. *Cancer Res* 1994, **54**, 3379–3382.
24. Rhyu M-G, Park W-S, Meltzer SJ. Microsatellite instability occurs frequently in human gastric carcinoma. *Oncogene* 1994, **9**, 29–32.
25. Suzuki H, Harpaz N, Tarmin L, *et al.* Microsatellite instability in ulcerative colitis-associated colorectal dysplasias and cancer. *Cancer Res* 1994, **54**, 4841–4844.
26. Lothe RA, Andersen SN, Hofstad B, *et al.* Deletion of 1p loci and microsatellite instability in colorectal polyps. *Genes Chromosomes Cancer* 1995, **14**, 182–188.
27. Shibata D, Peinado MA, Ionov Y, Malkhosyan S, Perucho M. Genomic instability in repeated sequences is an early somatic event in colorectal tumourigenesis that persists after transformation. *Nature Genet* 1994, **6**, 273–281.
28. Vermiglio F, Schlumberger M, Lazar V, Lefreré I, Bressac B. Absence of microsatellite instability in thyroid carcinomas. *Eur J Cancer* 1995, **31**, 128.
29. Horii A, Han H-J, Shimada M, *et al.* Frequent replication errors at microsatellite loci in tumours of patients with multiple primary cancers. *Cancer Res* 1994, **54**, 3373–3375.
30. Mullenbach R, Lagoda PJL, Welter C. An efficient salt-chloroform extraction of DNA from blood and tissues. *Trends Genet* 1989, **5**, 391.
31. Hedinger C, Williams ED, Sobin LH, eds. *Histologic Typing of Thyroid Tumours*, 2nd edn. World Health Organization—International Histologic Classification of Tumours. Berlin, Springer–Verlag, 1988.
32. Rosai J, Carcangiu ML, Delellis RA. Tumours of the thyroid gland. In *Atlas of Tumour Pathology*, 3rd series. Washington, DC, Armed Forces Institute of Pathology, 1992.
33. Hovig E, Smith-Sørensen B, Brøgger A, Børresen A-L. Constant denaturant gel electrophoresis, a modification of denaturing gel electrophoresis, in mutation detection. *Mutat Res* 1991, **262**, 63–71.
34. Børresen AL, Lothe RA, Meling GI, *et al.* Somatic mutations in the hMSH2 gene in microsatellite unstable colorectal carcinomas. *Human Mol Genet* 1995, **4**, 2065–2072.
35. Brentnall TA, Crispin DA, Bronner MP, *et al.* Microsatellite instability in nonneoplastic mucosa from patients with chronic ulcerative colitis. *Cancer Res* 1996, **56**, 1237–1240.
36. Thrash-Bingham CA, Salazar H, Freed JJ, Greenberg RE, Tartof KD. Genomic alterations and instabilities in renal cell carcinomas and their relationship to tumor pathology. *Cancer Res* 1995, **55**, 6189–6195.
37. Weber JL, Wong C. Mutation of human short tandem repeats. *Human Mol Genet* 1993, **2**, 1123–1128.
38. Field JK, Kiaris H, Howard P, Vaughan ED, Spandidos DA, Jones AS. Microsatellite instability in squamous cell carcinoma of the head and neck. *Br J Cancer* 1995, **71**, 1065–1069.
39. Huddan RA, Wooster R, Horwic A, Cooper CS. Microsatellite instability in human testicular germ cell tumours. *Br J Cancer* 1995, **72**, 642–645.
40. Loeb LA. Mutator phenotype may be required for multistage carcinogenesis. *Cancer Res* 1991, **51**, 3075–3079.
41. Kopp P, Kimura ET, Aeschimann S, *et al.* Polyclonal and monoclonal thyroid nodules coexist within human multinodular goitres. *J Clin Endocrinol Metab* 1994, **79**, 134–139.
42. Strand M, Prolla TA, Liskay RM, Petes TD. Destabilization of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair. *Nature* 1993, **365**, 274–276.
43. Bhattacharyya P, Skandalis A, Ganesh A, Groden J, Meuth M. Mutator phenotypes in human colorectal carcinoma cell lines. *Proc Natl Acad Sci USA* 1994, **91**, 6319–6323.
44. Liu B, Nicolaides NC, Markowitz S, *et al.* Mismatch repair gene defects in sporadic colorectal cancers with microsatellite instability. *Nature Genet* 1995, **9**, 48–55.

Acknowledgements—The authors thank Dra. Elsa Fonseca for her help in the histological evaluation of the cases. This work was supported by JNICT grant (PECS/C/SAU/254/95) and a grant from the Norwegian Cancer Society.

PAPER V

Int. J. Cancer: **70,** 32–38 (1997)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

E-CADHERIN GENE ALTERATIONS ARE RARE EVENTS IN THYROID TUMORS

Paula SOARES[1], Geert BERX[2], Frans VAN ROY[2] and Manuel SOBRINHO-SIMÕES[1]*

[1]*IPATIMUP, Department of Pathology, Porto, Portugal*
[2]*Department of Molecular Biology, University of Ghent and VIB, Belgium*

Reduced expression of E-cadherin has been associated with loss of differentiation and/or increased invasiveness and metastatic ability in several types of neoplasms. Rare studies on thyroid tumors have shown minimal or absent expression in undifferentiated carcinomas and a variable degree of immunoreactivity/mRNA expression in follicular and papillary carcinomas. We studied immunohistochemically 2 follicular adenomas, 8 follicular carcinomas, 18 papillary carcinomas and 3 poorly differentiated carcinomas to evaluate E-cadherin expression. In 7 papillary carcinomas, lymph node metastases were also studied. The *E-cadherin* gene was analysed in 27 tumors by PCR/SSCP followed, whenever appropriate, by DNA sequencing. LOH at the *E-cadherin* locus was assessed in 16 tumors. Reduced and heterogeneous expression of E-cadherin was found in primary and metastatic papillary carcinomas, whereas follicular carcinomas showed moderate to strong homogeneous immunoreactivity, and poorly differentiated carcinomas showed no or very faint immunoreactivity. The only case harbouring a (missense type) mutation of the *E-cadherin* gene was a papillary carcinoma exhibiting immunoreactivity in the primary tumor as well as in metastasis. LOH was found in a follicular adenoma and in a poorly differentiated carcinoma without evidence of mutation in the remaining allele. Our results show that irreversible alterations (allele loss or mutation) of the *E-cadherin* gene are infrequent in thyroid tumors. The reduced and heterogeneous expression of E-cadherin in thyroid carcinomas, particularly of the papillary histotype, appears to reflect transcriptional regulation or post-transcriptional modulation rather than structural abnormalities. *Int. J. Cancer,* 70:32–38, 1997.

Cadherins, the Ca^{2+}-dependent cell adhesion family of molecules, are key molecules in the cell junctional complexes (Gumbiner *et al.*, 1988). They mediate specific cell-cell association by homophilic interactions (Kemler, 1993). The cadherins are transmembrane glycoproteins presenting in the extracelular domain a characteristic tandem repeated sequence ("cadherin domains") which confers adhesive specificity (Shapiro *et al.*, 1995). The function of cadherins also depends on their cytoplasmatic binding domain which connects them with the cytoskeleton via complexes with catenins (Kemler, 1993).

Reduction and/or loss of expression of E-cadherin have been observed in several types of carcinomas (Shiozaki *et al.*, 1991; Brabant *et al.*, 1993; Sorscher *et al.*, 1995). These changes are particularly prominent in poorly differentiated, undifferentiated and/or invasive tumors. Loss of expression of E-cadherin has been associated with mutation of the *E-cadherin* gene and loss of heterozygosity (LOH) in 16q22 in infiltrative lobular carcinomas of the breast (Berx *et al.*, 1995). Becker *et al.* (1994) described a gastric carcinoma presenting LOH with retention of a mutated *E-cadherin* allele. These findings support a role of *E-cadherin* as a tumor and/or invasion suppressor gene, as previously suggested by *in vitro* observations (Vleminckx *et al.*, 1991).

Expression of E-cadherin in normal thyroid and in a small series of thyroid tumors was first described by Eidelman *et al.* (1989). Brabant *et al.* (1993) studied a larger series of thyroid tumors and found minimal or absent expression in undifferentiated carcinomas, in contrast with a variable degree of immunoreactivity/mRNA expression in the differentiated histotypes. Brabant *et al.* (1993) concluded that loss of gene expression and/or defective post-transcriptional control of E-cadherin appear to be restricted to undifferentiated and metastatic thyroid tumors.

To find out whether the variable expression of E-cadherin in differentiated malignant thyroid tumors may be related to irreversible abnormalities in the *E-cadherin* gene, we undertook the present immunohistochemical and molecular biology study of E-cadherin in several types of thyroid lesions.

MATERIAL AND METHODS

Tumor specimens

Thirty-one thyroid tumors were retrieved from the files of the Unit of Molecular Pathology of IPATIMUP. The classification of the lesions was made according to Hedinger *et al.* (1988) in follicular adenoma (n = 2), follicular carcinoma (n = 8), papillary carcinoma (n = 18) and poorly differentiated carcinoma (n = 3) in sections stained with haematoxylin and eosin. In 7 of the cases of papillary carcinoma, lymph node metastases were also studied; in the remaining 11 cases of papillary carcinoma no involvement of the regional lymph nodes was noticed at surgery. Seven of the follicular carcinomas were of the minimally invasive type (1 composed of Hürthle cells), and the remaining tumor was a widely invasive follicular carcinoma. The histologic subtypes of the 18 papillary carcinomas as follows: common type, 13 cases (1 displaying Hürthle cell features); follicular variant, 3 cases; micropapillary carcinoma, 1 case, and diffuse sclerosing variant, 1 case. Foci of squamous metaplasia were observed in 3 papillary carcinomas. In 23 cases, adjacent normal-looking thyroid parenchyma could be identified and was therefore used as an internal control. In the remaining 8 cases the adjacent parenchyma displayed lymphocytic thyroiditis (5 cases) and nodular goitre (3 cases, 2 of which also displayed papillary hyperplasia).

Immunohistochemistry

Immunostaining of E-cadherin was performed using the avidin-biotin-peroxidase (ABC) complex as described by Holm *et al.* (1985) with an additional step for microwave antigen retrieval. Sections (5 μm) were cut and mounted on gelatin-coated slides. Antigen retrieval was done by microwave treatment 5 × 1.5 min at 750 W in a 0.1% detergent solution (Hazelbag *et al.*, 1995). After cooling (20 min at room temperature), the sections were immersed in 0.3% hydrogen peroxide (H_2O_2) in methanol to block endogenous peroxidase activity. Non-specific staining was eliminated by 20 min of incubation with normal rabbit serum. Excess normal serum was removed, replaced by the anti-human E-cadherin

Contract grant sponsor: Praxis XXI; contract grant number: 212.1/BIA/100/94

*Correspondence to: the IPATIMUP, Department of Pathology, Medical Faculty of Porto, Hospital S. João, P-4200 Porto, Portugal. Fax: 351 2 5503940.

Received 4 July 1996; revised 11 September 1996.

antibody diluted 1:300 (HECD-1, Zymed, San Francisco, CA) and incubated overnight at 4°C. After washing the slides, sections were incubated with a 1:200 dilution of biotin-labelled secondary antibody followed by ABC complex (avidin 1:100 and biotin-labelled peroxidase 1:100; Dako, Glostrup, Denmark) for 30 min each. Subsequently, sections were stained for 7 min with 0.05% 3,3-diaminobenzidine tetrahydrochloride, 0.01% H_2O_2 in 0.05 M Tris/HCl buffer, pH 7.6, counterstained with hematoxylin, dehydrated and mounted. Normal serum was diluted in 25% BSA in TBS, and primary antibodies, biotinylated secondary antibodies and ABC were diluted in 12.5% BSA in TBS.

Seven cases of papillary carcinoma presenting heterogeneous immunoreactivity for E-cadherin were selected for a systematic immunohistochemical study using serial sections. Antisera for thyroglobulin (1/1,000), cytokeratins (AE1/AE3, 1/50 and CAM 5.2, 1/10), vimentin (1/80), Tn antigen (5F4, 1/15), sialosyl-Tn antigen (3F1, 1/8), T antigen (3C9 1/10, without and after neuraminidase treatment) and MIB-1 (1/100) were investigated. Positive controls were included in all series for all the antibodies used. As negative controls, immunoglobulins of the same subclasses and in the same protein concentrations as the primary specific antibodies were used.

The results were scored semi-quantitatively by percentage of immunopositive cells and intensity of staining (0, absent; 0/+, very faint stain; +, faint; ++, moderate; and +++, strong staining). Localization of the staining (membranous and/or cytoplasmatic) was also evaluated in every case.

Analysis of the E-cadherin *gene by PCR/SSCP*

High m.w. DNA was extracted according to a standard procedure (Mullenbach *et al.*, 1989) from 27 cases. (Frozen material from 4 papillary carcinomas was not available.) PCR reactions were performed in 25 µl volumes of a mixture containing: 2.5 µl of 10× Taq polymerase buffer, 200 µM of each deoxynucleotide triphosphate except dCTP, 20 µM of dCTP, 0.5 µCi of [α-^{32}P] dCTP, 2–3 mM $MgCl_2$, 25 pmol of each primer and 250 ng of genomic DNA. Mineral oil overlaid the PCR reaction. After a denaturation step for 2 min at 95°C, Taq polymerase (0.2 U) was added through the oil of each tube at 80°C. Thirty-five cycles of 30 sec at 94°C, 30 sec at the appropriate annealing temperature and 45 sec at 72°C were performed with a final extension step of 72°C for 10 min on a thermal cycler. The sequence of the primers used for each exon of the *E-cadherin* gene, the annealing temperatures, the $MgCl_2$ concentrations and the specific conditions were as previously described (Berx *et al.*, 1995). PCR reaction products were diluted 1:1 with a loading buffer (95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol) and denaturated at 99°C for 2 min. Electrophoresis of 2 µl of the denaturated PCR products was carried out in non-denaturing gels (5% acrylamide with 2% cross-linking), either without glycerol (8°C) or with 5% glycerol (20°C), and run at 3 W overnight in 0.5× TBE running buffer. The gels were blotted onto 3MM Whatman paper, dried for 30 min on a vacuum gel dryer and exposed to Hyperfilm (Amersham, Aylesbury, UK) overnight at room temperature.

Sequencing

The bands presenting mobility shifts as well as the corresponding standard bands were excised from the paper, eluted in 150 µl water by incubating for 30 min at 65°C and then kept for 15 min at −70°C (this procedure was performed 3 times). Ten microliters of this solution were used for reamplification as described above, but under non-radioactive conditions. Five microliters of the PCR products were subsequently subjected to enzymatic treatment (1 U exonuclease and 1 U shrimp alkaline phosphatase) to destroy the residual primers and nucleotides (Sequenase PCR Product Sequencing Kit, Amersham). Sequenase 2.0 (USB, Cleveland, OH) was used to determine the sequence of both strands, using either one of the original PCR primers.

Loss of heterozygosity

The LOH study was done on 16 tumor samples (2 follicular adenomas, 3 follicular carcinomas, 9 papillary carcinomas and 2 poorly differentiated carcinomas) and on matched constitutional DNA. Two microsatellite markers were used, D16S265 and D16S301 (Human Gene Mapping kit, ISOGEN, Amsterdam, Netherlands), and the PCR procedures were done according to the manufacturer's indications. The annealing temperature for both markers was 58°C. After PCR, the products were run in a 6% denaturating gel, and exposed to Hyperfilm (Amersham) overnight at room temperature.

RESULTS

Immunohistochemistry

The immunohistochemical results are summarized in Tables I and II.

TABLE I – E-CADHERIN IMMUNOHISTOCHEMICAL EXPRESSION IN THYROID TUMORS[1]

Case	Lesion	Sex/Age (yr)	Immunohistochemistry		
			Membrane	Cytoplasm	Approx. percentage of cells
1	FA	F/25	++	0	100
2	FA	F/35	++	++	100
3	PC, FV	F/45	++	0	80
4	PC	F/49	ND[2]	ND	ND
5	PC	F/42	0/+	+	100
5	Met		0/+	+	100
6	PC	F/40	0/+	++	80
7	PC	F/32	++	0	100
8	PC, DSV	F/40	+	+	100
8	Met		+	0/+	100
9	PC, FV	F/33	+++	++	70
10	PC	F/36	+	0	80
10	Met		+	0	<30
11	PC	F/41	++	+	80
12	PC	F/59	+	++	100
13	mPC	F/50	+	0/+	80
14	PC, FV	F/27	+	0/+	100
15	PC	F/46	+	++	70
16	PC	F/22	+	++	70
17	PC	F/35	+	0/+	70
17	Met		+	0/+	70
18	PC	F/38	+++	0/+	80
18	Met		0/+	0/+	50
19	PC	F/13	0/+	+	40
19	Met		0/+		<40
20	PC	M/66	+	0/+	80
20	Met		+	0/+	70
21	FC	F/54	+++	0	100
22	FCH	F/69	+++	++*	100
23	FC	F/56	++	+	80
24	FC	F/36	++	+	100
25	FC	F/29	+++	0	100
26	FC	F/57	+++	+	100
27	FC	F/34	++	0	100
28	FC	F/39	+++	0/+	100
29	PDC	M/81	0	+	100
30	PDC	F/68	0/+	0/+	100
31	PDC	F/32	0	0/+	100

[1]FA, follicular adenoma; FC, follicular carcinoma; PC, papillary carcinoma; PDC, poorly differentiated carcinoma; FCH, Hürthle cell follicular carcinoma; mPC, micropapillary carcinoma; FV, follicular variant of papillary carcinoma; DSV, diffuse sclerosing variant of papillary carcinoma; Met, metastasis; ND, not determined.

TABLE II – SUMMARY OF THE IMMUNOHISTOCHEMICAL RESULTS FOR E-CADHERIN IN THYROID TUMORS[1]

Tumor type (n)	Membrane					Cytoplasm				
	0	0/+	+	++	+++	0	0/+	+	++	+++
FA (2)				2		1			1[2]	
PC (17)[3]		3	9	3	2	3	6	3	5	
FC (8)				3	5	3	1	3	1[4]	
PDC (3)	2		1				2	1		
Met (7)		3	4			1	5	1		

[1]FA, follicular adenoma; FC, follicular carcinoma; PC, papillary carcinoma; PDC, poorly differentiated carcinoma; Met, metastasis.–[2]Adenoma with prominent papillary hyperplasia.–[3]One case was excluded for technical reasons.–[4]Hürthle cell carcinoma.

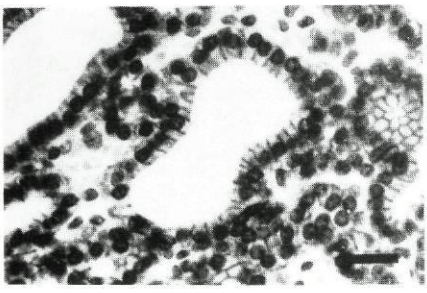


FIGURE 1 – Follicular carcinoma displaying strong membrane immunoreactivity (case 28). Scale bar = 25 µm.

Normal thyroid. Membrane immunoreactivity was observed in the follicular cells of the normal parenchyma, being less intense in flat cells lining large follicles than in cuboidal or tall cells lining medium-sized or small follicles. The lateral border of the cells presented more intense staining than the basal border, and the immunoreactivity in the apical border was faint or absent.

Goitre and lymphocytic thyroiditis. The pattern of immunoreactivity in goitres, with or without papillary hyperplasia, was similar to that of normal thyroid. The same holds true for lymphocytic thyroiditis except for the more intense immunoreactivity of the follicular cells that were present amidst the lymphoid infiltrates.

Follicular adenomas. The pattern of immunoreactivity in one of the adenomas was similar to that of normal thyroid, whereas in the adenoma which displayed papillary hyperplasia, cytoplasmatic staining was observed in addition to membrane immunoreactivity.

Follicular carcinomas. Immunoreactivity was homogeneous throughout the tumors, with moderate to strong (scores ++ and +++) intensity. Staining was seen in the membrane around the whole cells rather than being clearly polarized as in normal thyroid and benign conditions (Fig. 1). Some cytoplasmatic staining was observed in addition to membrane immunoreactivity.

Papillary carcinomas. Immunoreactivity was generally less intense in papillary carcinomas than in follicular carcinomas. Also at variance with follicular carcinomas, immunoreactivity for E-cadherin was clearly heterogeneous in papillary carcinomas, *i.e.*, positive areas co-existed side by side with negative areas (Fig. 2). As in follicular carcinomas, the polarized expression of E-cadherin was partly lost. Cytoplasmic staining predominated in cases exhibiting a pure papillary pattern (Fig. 3), whereas in cases presenting a follicular pattern membrane staining was more intense.

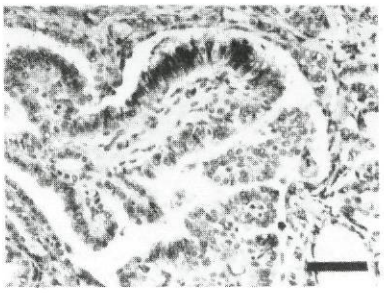


FIGURE 2 – Papillary carcinoma presenting heterogeneous immunoreactivity (case 10). Scale bar = 35 µm.

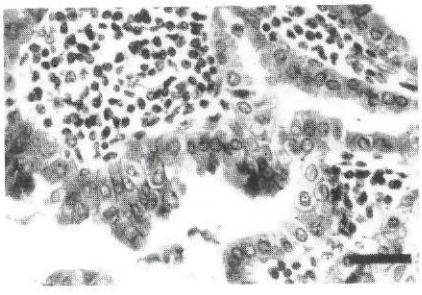


FIGURE 3 – Strong cytoplasmatic staining in a papillary carcinoma (case 12). Scale bar = 17 µm.

Comparison of results obtained in primary tumors with their respective metastases yielded similar staining scores in 6 cases. In the remaining case (case 18), the metastasis displayed decreased intensity of staining. Comparison of the papillary carcinomas which had lymph node involvement with those in which there was no apparent metastatic involvement did not disclose any major difference in intensity of immunoreactivity and percentage of immunoreactive cells.

No consistent correlation was observed between heterogeneity of E-cadherin immunoreactivity in some papillary carcinomas and other immunohistochemical markers except for cytokeratin CAM 5.2 which showed a pattern of immunoreactivity similar to that of E-cadherin: foci displaying more intense immunoreactivity for E-cadherin also displayed more intense immunoreactivity for cytokeratins.

Poorly differentiated carcinomas. Two of the 3 poorly differentiated carcinomas showed a (very) faint immunoreactivity. In the remaining case, strong membrane positivity was seen in the well-differentiated areas of the co-existing follicular carcinoma, whereas the less differentiated, solid areas presented a (very) faint immunoreactivity (Fig. 4).

SSCP/Sequencing

The 16 exons of the *E-cadherin* gene were analysed by PCR/SSCP in 27 cases. Gene polymorphisms were detected in a papillary carcinoma (case 5; n = 1) and a follicular carcinoma

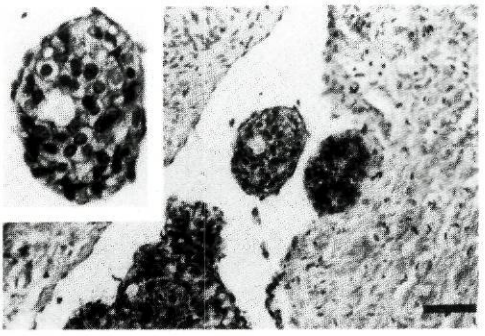


FIGURE 4 – Poorly differentiated carcinoma displaying membrane staining in the neoplastic cells that invaded a large vessel (case 30). Inset: Higher magnification of the neoplastic cluster within the vessel. Scale bar = 35 µm (magnification of the inset is twice as great).

(case 25; n = 2) (Table III). In a diffuse sclerosing variant of papillary carcinoma (case 8) there was a mobility shift in the product of amplicon 12 which was restricted to the tumor (not present in the corresponding constitutional control); on DNA sequencing, this shift could be attributed to a missense mutation in codon 592, leading to a non-conservative amino acid change of Alanine-Threonine (Fig. 5, Table III). The E-cadherin staining pattern of this case did not differ from those of the other papillary carcinomas of the series: there was no polarized expression of E-cadherin, and the staining was both cytoplasmatic and membranous (Fig. 6).

Loss of heterozygosity

Two of the 16 cases analysed were not informative for the markers used in the study. One follicular adenoma (case 2) and a poorly differentiated carcinoma (case 29) showed LOH for D16S301 and D16S265, respectively (Table III). All the other cases were informative for at least one of the markers, and no evidence of LOH was detected.

DISCUSSION

In agreement with previously reported data (Eidelman *et al.*, 1989; Brabant *et al.*, 1993), we observed expression of E-cadherin in normal follicular cells, the tall cells showing a more clear-cut membranous staining than the flat cells lining large follicles. The tumor-like lesions and one of the adenomas reacted like the normal thyroid tissues.

Distinct patterns of reactivity were found in the 2 differentiated carcinoma types: follicular carcinomas presented a moderate to strong membranous immunoreactivity in every neoplastic cell, whereas in papillary carcinomas the immunoreactivity was comparatively less intense and negative areas were frequently observed. Moreover, in pure papillary areas the cytoplasmatic staining appeared to be more intense than in areas with follicular structure. This result confirms those of Eidelman *et al.* (1989), who found that non-polarized expression of cell-CAM 120/80 (identical to E-cadherin) was not only observed in undifferentiated carcinomas, but also in cells arranged into distinct histologic structures (cords, papillae, or gland-like formations).

The low and heterogeneous expression of E-cadherin in papillary carcinomas confirms the results of Brabant *et al.* (1993), who observed a reduction of E-cadherin mRNA in a majority of papillary carcinomas, in comparison with normal thyroid, and a strong intercellular variation of immunofluorescence in most of the papillary carcinomas of their series.

We cannot rule out, on the other hand, a putative role for a post-transcriptional mechanism(s). Indeed, our results fit conform to the pericellular redistribution and cytoskeletal disconnection of the E-cadherin-catenin complex observed in thyroid carcinomas compared with the normal and adenomatous tissues described by Serini *et al.* (1996), according to whom, the pericellular redistribution of E-cadherin (that we observed mostly in papillary carcinomas) might be due to decreased thyrosine phosphorylation of the E-cadherin cytodomain.

In the 7 metastases of papillary carcinomas that we analysed, E-cadherin expression was of the same intensity or only slightly lower than in the respective primary tumors. Similar (or slightly reduced) expression of E-cadherin in metastases and matched primary tumors has been reported by others in different organs (Shimoyama *et al.*, 1989; Sorscher *et al.*, 1995). The papillary carcinomas in our series that presented metastases did not differ significantly from those without apparent metastases. Our results are thus in contrast to those of Brabant *et al.* (1993), who observed lower or undetectable E-cadherin immunoreactivity in tumors presenting lymph node metastases, distant metastases or local recurrence. It remains to be seen whether there is any relationship between the heterogeneous and relatively low level of expression of E-cadherin in most papillary carcinomas and their well-known tendency toward local invasiveness and lymphatic dissemination.

In follicular carcinomas immunoreactivity was moderate to strong and homogeneous throughout the tumors. These results are in accordance with the results obtained at the mRNA level by Brabant *et al.* (1993), who reported high levels of mRNA in follicular carcinomas, but observed some discrepancies in the results obtained by Northern and immunofluorescence methods.

The only case harbouring a mutation of the *E-cadherin* gene was a papillary carcinoma displaying immunoreactivity in the primary tumor as well as in the lymph node metastasis. Becker *et al.* (1994) also detected, in a series of gastric carcinomas, immunoreactivity in tumors presenting alterations at the DNA level. The missense mutation present in case 8 led to the substitution of an amino acid residue that apparently did not interfere with expression of the recognition site of the HECD-1 antibody. In this case, the pattern of E-cadherin immunoreactivity (cytoplasmic and membrane staining without polarization) did not differ from that observed in other papillary carcinomas.

Our results show that both LOH and mutations at the *E-cadherin* locus are rare in well-differentiated thyroid carcinomas, thus leading to the conclusion that the peculiar downregulated expression of E-cadherin seen in papillary carcinomas cannot be ascribed to any of these events.

Our data on poorly differentiated carcinomas, although quite limited, confirm a reduction or absence of E-cadherin expression in poorly differentiated tumors, as reported by several authors in systems other than thyroid (Shiozaki *et al.*, 1991; Brabant *et al.*, 1993; Sorscher *et al.*, 1995). In one case of poorly differentiated carcinoma negative for E-cadherin immunoreactivity, LOH was detected, but without evidence of mutation using SSCP. Reduction of gene dosage due to allelic loss could result in reduced gene expression, as suggested by Umbas *et al.* (1992). The number of poorly differentiated carcinomas analysed in our series is too small to allow a valid evaluation of the general occurrence of LOH and/or mutations at the *E-cadherin* locus in these tumors. Furthermore, the poorly differentiated carcinoma with LOH occurred in an elderly patient and may well merely be secondary in neoplastic development. Regardless of the mechanism(s) underlying the loss of

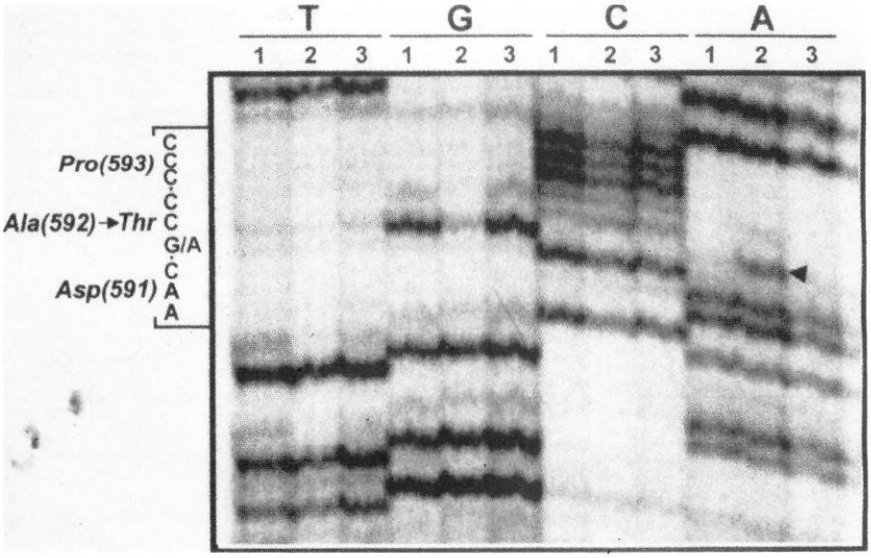


FIGURE 5 – Sequence analysis of a tumor-restricted abnormally shifted PCR/SSCP band identified in case 8. Samples with the normal sequence were loaded in lanes 1 and 3. Arrowhead points at the mutated nucleotide.

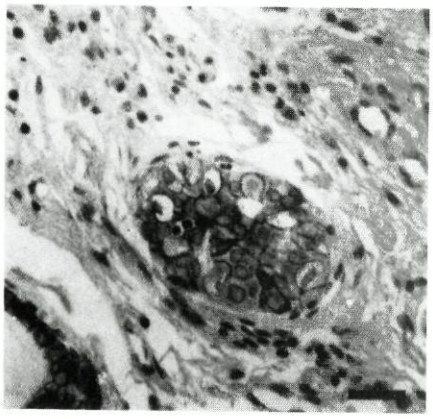


FIGURE 6 – Cytoplasmatic and membrane staining in the diffuse sclerosing variant of papillary carcinoma that harboured the missense mutation depicted in Figure 5 (case 8). Scale bar = 16 μm.

TABLE III – E-CADHERIN GENE ALTERATIONS AND LOH RESULTS[1]

Case	Lesion	LOH		SSCP/sequencing		
		D16S 265	D16S 301	Exon	Codon	Change
2	FA	–	LOH			—
5	PC	ND	ND	9	404	CCC → CCG Pro → Pro
8	PC	+	–	12	592	GCC → ACC Ala → Thr
25	FC	+	+	12	632	CAC → CAT Hist → Hist
				16	878	GGC → GGT Gly → Gly
29	PDC	LOH	–			—

[1]LOH, loss of heterozygosity; +, informative; –, not informative; FA, follicular adenoma; FC, follicular carcinoma; PC, papillary carcinoma; PDC, poorly differentiated carcinoma; ND, not done.

E-cadherin expression in poorly differentiated and undifferentiated tumors of the thyroid or of other organs, a common relationship among the following features becomes apparent: reduçed E-cadherin expression, reduced differentiation and increased aggressiveness of the tumors. The finding of foci of poorly differentiated carcinoma with extremely reduced E-cadherin expression on a background of follicular carcinoma displaying fairly intense immunoreactivity for E-cadherin (case 30) supports the existence of a close link between differentiation and E-cadherin expression but does not allow the establishment of a timing for the process of dedifferentiation.

Treatment of an oesophageal cell line (TE-2R) with epidermal growth factor (EGF) induces morphological changes in the cells, with sparse colonies and invasive behaviour in organotypic raft culture (Shiozaki *et al.*, 1995). These changes are accompanied by a change in the localisation of E-cadherin from the cell borders to the whole cell surface. Shiozaki *et al.* (1995) also reported tyrosine phosphorylation of β-catenin (which suppresses cadherin function *in vitro*) in cells treated with EGF and suggested that EGFR could be the candidate kinase responsible for tyrosine phosphorylation of β-catenin and for consequent impairment of the E-cadherin cell-cell adhesion system. Thyroid cell lines derived from papillary and

follicular carcinomas show enhanced migration and invasion after treatment with EGF, while the tumorigenic capacity of a papillary cell line became higher after treatment with EGF (Hoelting *et al.*, 1994). It has also been reported that transforming growth factor-α (TGF-α), another ligand of the EGFR, induces a motile fibroblast-like phenotype *in vitro* (Gavrilovic *et al.*, 1990). The co-expression of EGFR and TGF-α in papillary carcinomas has been reported as being compatible with the existence of an autocrine loop in these tumors (Lemoine *et al.*, 1990; Haugen *et al.*, 1993). Taken together, these data suggest that heterogeneous and non-polarized expression of E-cadherin in papillary carcinomas could be related to expression of EGFR/TGF-α. Furthermore, Akslen and Varhaug (1995) found a significant association between cytoplasmic expression of EGFR and extrathyroidal growth of the primary tumor, recurrence and poor prognosis; it would be interesting to examine whether these features may be related to E-cadherin down-regulation.

Other growth factors have been implicated in the regulation of the expression of E-cadherin. The HGF/SF enhances dissociation and motility of epithelial cells, and the corresponding receptor (c-MET) has been found to be overexpressed in thyroid tumors (Di Renzo *et al.*, 1992). Marked scattering of cells of gastric cell lines treated with HGF/SF was associated with reduction of the expression of P- and E-cadherin (Tannapfel *et al.*, 1994). The regulation of cell adhesion in these gastric cell lines is accomplished at the protein level since no alteration at the mRNA level of cadherins has been detected (Tannapfel *et al.*, 1994).

Thyroid tumors overexpressing c-MET include mainly the histotype variants that appear to be correlated with guarded prognosis and frequently give rise to locally advanced disease and/or distant metastases (Di Renzo *et al.*, 1992). The enhanced invasive potential and aggressive features of the tumors overexpressing c-MET may also be related to down-regulation of E-cadherin, but the exploitation of this hypothesis rests beyond the scope of the present study.

In summary, our results show that irreversible alterations of the *E-cadherin* gene are infrequent in thyroid carcinomas, thus supporting the assumption that the decreased and heterogeneous expression of E-cadherin in thyroid carcinomas, particularly in the papillary histotype, reflects transcriptional regulation or post-transcriptional modulation rather than structural abnormalities. We suggest, furthermore, that such modulation depends on alterations in the expression of growth factors/growth factor receptors. We can not rule out, alternatively, a putative hypermethylation of the *E-cadherin* promoter region with consequent silencing of the gene (Yoshiura *et al.*, 1995), a mechanism which has been reported in tumors of other organs (Graff *et al.*, 1995).

ACKNOWLEDGEMENTS

The authors thank Mrs. D. Leitão for her help with the immunohistochemical study. G.B. is a fellow of the VIB, Flanders, and F.v.R. is Research Director, National Fund for Scientific Research (NFSR), Belgium and is supported by the Belgium Cancer Association and the NFSR, Belgium.

REFERENCES

AKSLEN, L.A. and VARHAUG, J.E., Oncoproteins and tumour progression in papillary thyroid carcinoma. *Cancer,* **76,** 1643–1654 (1995).

BECKER, K.-F., ATKINSON, M.J., REICH, U., BECKER, I., NEKARDA, H., SIEWERT, J.R. and HÖFLER, H., E-cadherin gene mutations provide clues to diffuse type gastric carcinomas. *Cancer Res.,* **54,** 3845–3852 (1994).

BERX, G., CLETON-JANSEN, A.M., NOLLET, F., LEEUW, W.J.F., VAN DE VIJVER, M.J., CORNELISSE, C. and VAN ROY, F., E-cadherin is a tumour/invasion suppressor gene mutated in lobular breast cancers. *EMBO J.,* **14,** 6107–6115 (1995).

BRABANT, G., HOANG-VU, C., CETIN, Y., DRALLE, H., SCHEUMANN, G., MÖLNE, J., HANSSON, S., ERICSON, L.E. and NILSSON, M., E-cadherin: a differentiation marker in thyroid malignancies. *Cancer Res.,* **53,** 4987–4993 (1993).

DI RENZO, M.F., OLIVERO, M., FERRO, S., PRAT, M., BONGARZONE, I., PILOTTI, S., BELFIORE, A., CONSTANTINO, A., VIGNERI, R., PIEROTTI, M.A. and COMOGLIO, P.M., Overexpression of the c-MET/HGF receptor gene in human thyroid carcinomas. *Oncogene,* **7,** 2549–2553 (1992).

EIDELMAN, S., DAMSKY, C.H., WHEELOCK, M.J. and DAMJANOV, I., Expression of the cell-cell adhesion glycoprotein cell-CAM 120/80 in normal human tissues and tumours. *Amer. J. Pathol.,* **135,** 101–110 (1989).

GAVRILOVIC, J., MOENS, G., THIERY, J.P. and JOUANNEAU, J., Expression of transfected transforming growth factor alpha induces a motile fibroblast like phenotype with extracellular matrix-degrading potential in a rat bladder carcinoma cell line. *Cell Regul.,* **1,** 1003–1014 (1990).

GRAFF, J.R., HERMAN, J.G., LAPIDUS, R.G., CHOPRA, H., XU, R., JARRARD, D.F., ISAACS, W.B., PITHA, P.M., DAVIDSON, N.E. and BAYLIN, S.B., E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. *Cancer Res.,* **55,** 5195–5199 (1995).

GUMBINER, B., STEVENSON, B. and GRIMALDI, A., The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. *J. Cell Biol.,* **107,** 1575–1587 (1988).

HAUGEN, D.R., AKSLEN, L.A., VARHAUG, J.E. and LILLEHAUG, J.R., Demonstration of a TGF-alpha -EGF-receptor autocrine loop and c-myc protein overexpression in papillary thyroid carcinomas. *Int. J. Cancer,* **55,** 37–43 (1993).

HAZELBAG, H.M., BROEK, L.J.C.MVD., DORST, E.B.LV., OFFERHAUS, G.J.A., FLEUREN, G.J. and HOGENDOORN, P.C.W., Immunostaining of chain-specific keratins on formalin-fixed, paraffin-embedded tissues: a comparison of various antigen retrieval systems using microwave heating and proteolytic pre-treatments. *J. Histochem. Cytochem.,* **43,** 429–437 (1995).

HEDINGER, C., WILLIAMS, E.D. and SOBIN, L.H., (eds.), *Histologic typing of thyroid tumours* (2nd ed.), World Health Organization. International Histologic Classification of Tumors. Springer, Berlin (1988).

HOELTING, T., SIPERSTEIN, A.E., CLARK, O.H. and DUH, Q-Y., Epidermal growth factor enhances proliferation, migration, and invasion of follicular and papillary thyroid cancer *in vitro* and *in vivo. J. Clin. Endocrinol. Metab.,* **79,** 401–408 (1994).

HOLM, R., SOBRINHO-SIMÕES, M., NESLAND, J.M., GOULD, V.E. and JOHANNESSEN, V., Medullary carcinoma of the thyroid gland. An immunohistochemical study. *Ultrastruct. Pathol.,* **8,** 25–41 (1985).

KEMLER, R., From cadherins to catenins: cytoplasmic interactions and regulation of cell adhesion. *Trends Genet.,* **9,** 317–321 (1993).

LEMOINE, N.R., WYLLIE, F.S., LILLEHAUG, J.R., STADDON, S.L., HUGHES, C.M., AASLAND, R., SHAW, J., VARHAUG, J.E., BROWN, C.L., GULLICK, W.J. and WYNFORD-THOMAS, D., Absence of abnormalities of the c-erbB-1 and c-erbB-2 proto-oncogenes in human thyroid neoplasia. *Europ. J. Cancer,* **26,** 777–779 (1990).

MULLENBACH, R., WELDAR, C. and LAGODA, P., An efficient salt chloroform extraction of DNA from blood and tissues. *Trends Genet.,* **5,** 391 (1989).

SERINI, G., TRUSOLINO, E.S., CREMONA, O., ROSSI, M., ANGELI, A., ORLANDI, F. and MARCHISO, P.C., Changes in integrin and E-cadherin expression in neoplastic versus normal thyroid tissue. *J. nat. Cancer Inst.,* **88,** 442–449 (1996).

SHAPIRO, L., FANNON, A.M., KWONG, P.D., THOMPSON, A., LEHMANN, M.S., GRÜBEL, G., LEGRAND, J-F., ALS-NIELSEN, J., COLMAN, D.R. and HENDRICKSON, WA., Structural basis of cell-cell adhesion by cadherins. *Nature (Lond.),* **374,** 327–337 (1995).

SHIMOYAMA, Y., HIROHASHI, S., HIRANO, S., NOGUCHI, M., SHIMOSATO, Y., TAKEICHI, M. and ABE, O., Cadherin cell-adhesion molecules in human epithelial tissues and carcinomas. *Cancer Res.,* **49,** 2128–2133 (1989).

SHIOZAKI, H., KADOWAKI, T., DOKI, Y., INOUE, M., TAMURA, S., OKA, H., IWAZAWA, T., MATSUI, S., SHIMAYA, K., TAKEICHI, M. and MORI, T., Effect of epidermal growth factor on cadherin-mediated adhesion in a human oesophageal cancer cell line. *Brit. J. Cancer,* **71,** 250–258 (1995).

SHIOZAKI, H., TAHARA, H., OKA, H., MIYATA, M., KOBAYASHI, K., TAMURA, S., IIHARA, K., DOKI, Y., HIRANO, S., TAKEICHI, M. and MORI, T., Expression of immunoreactive E-cadherin adhesion molecules in human cancers. *Amer. J. Pathol.*, **139,** 17–23 (1991).

SORSCHER, S.M., RUSSACK, V., CAGLE, M., FERAMISCO, J.R. and GREEN, M.R., Immunolocalization of E-cadherin in human head and neck cancer. *Arch. Pathol. Lab. Med.*, **119,** 82–84 (1995).

TANNAPFEL, A., YASUI, W., YOKOZAKI, H., WITTEKIND, C. and TAHARA, E., Effect of hepatocyte growth factor on the expression of E- and P-cadherin in gastric carcinoma cell lines. *Virchows Archiv.*, **425,** 139–144 (1994).

UMBAS, R., SCHALKEN, J.A., AALDER, T.W., CARTER, B.S., KARTHAUS, H.F.M., SCHAAFSMA, H.E., DEBRUYNE, F.M.J. and ISAACS, W.B., Expression of the cellular adhesion molecule E-cadherin is reduced or absent in high-grade prostate cancer. *Cancer Res.*, **52,** 5104–5109 (1992).

VLEMINCKX, K., VAKAET, JR. L., MAREEL, M.M., FIERS, W. and VAN ROY, F., Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell*, **66,** 107–119 (1991).

YOSHIURA, K., KANAI, Y., OCHIAI, A., SHIMOYAMA, Y., SUGIMURA, T. and HIROHASHI, S., Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. *Proc. nat. Acad. Sci. (Wash.)*, **92,** 7416–7419 (1995).

PAPER VI

SPORADIC *ret*-REARRANGED PAPILLARY CARCINOMA OF THE THYROID: A SUBSET OF SLOW GROWING, LESS AGGRESSIVE THYROID NEOPLASMS?

PAULA SOARES[1], ELSA FONSECA[1], DAVID WYNFORD-THOMAS[2] AND MANUEL SOBRINHO-SIMÕES[1]*

[1]*IPATIMUP, Department of Pathology, Medical Faculty, University of Porto, Porto, Portugal*
[2]*CRC Thyroid Tumour Biology Research Group, Department of Pathology, University of Wales, College of Medicine, Cardiff, U.K.*

SUMMARY

Despite the large amount of information accumulated on the role played by *ret* activation in the oncogenesis of papillary thyroid carcinoma (PTC), the biological and clinical significance of such activation '*in vivo*' remains controversial. The aim of this study was to address some of the existing controversies by comparing two groups of unselected PTCs, one with and the other without *ret* rearrangement, with regard to several clinicopathological and biological features. Thirty-three PTCs were selected at random. *ret* rearrangement was found in eight cases (24·2 per cent) using Southern blot analysis. The mean age of the patients with tumours displaying *ret* rearrangement (28 ± 3·1 years) was significantly lower than that of the patients harbouring cases that did not present rearrangement (45 ± 2·9 years). The large majority of the tumours with *ret* rearrangement displayed a papillary or mixed follicular–papillary pattern and very low proliferative activity. *ret* rearrangement correlated significantly with decreased cytoplasmic expression of E-cadherin. No significant differences were found regarding the gender of the patients, tumour size, multicentricity, extrathyroidal growth, vascular invasion, lymphocytic infiltration, lymph node involvement or the expression of E-cadherin (membrane), c-erb-B2, c-met, Bcl-2, and vimentin. It is proposed that sporadic PTCs harbouring a *ret* rearrangement occur frequently as slow growing, papillary, or predominantly papillary tumours that do not usually progress towards less differentiated neoplasms representing what might be described as a Bonsai phenotype. © 1998 John Wiley & Sons, Ltd.

J. Pathol. 184: 000–000, 1998.

KEY WORDSTHYROID; PAPILLARY CARCINOMA; *ret* oncogene; MIB-1; E-cadherin; c-erb-B2; c-met; Bcl-2

INTRODUCTION

Papillary thyroid carcinoma (PTC) is the commonest type of thyroid tumour, representing about 70 per cent of all thyroid carcinomas.[1,2] PTCs usually carry a good prognosis (90–95 per cent survival at 5 years), despite often presenting lymph node metastases at diagnosis.[1,2]

In 1987, Fusco *et al.*,[3] using DNA transfection analysis, reported a putative 'new' oncogene activated in PTCs and their lymph node metastases. The same group later reported that activation of the PTC oncogene resulted from an intrachromosomal rearrangement of an unknown amino-terminal sequence, named H4, to the tyrosine kinase domain of the *ret* proto-oncogene.[4] Two other rearrangements of *ret* were subsequently identified in PTC: *ret*/PTC2, involving an RIa regulatory subunit of protein kinase A localized in chromosome 17,[5,6] and *ret*/PTC3, involving an intrachromosomal rearrangement with a previously unknown gene named *RFG*/*ELE1*.[5,7] Recently, different types of rearrangement involving *ELE1* and *ret* were identified in post-Chernobyl PTCs.[8,9]

The chimeric proteins encoded by the rearrangements lead to the relocation of the *ret* tyrosine kinase domain from the membrane to the cytoplasm, display an '*in vitro*' autophosphorylation activity, and are constitutively phosphorylated on tyrosine. Their activation may be mediated by the formation of homo- and heterodimers[9] mimicking activation by the physiological ligand, glial-cell line-derived neurotrophic factor (GDNF).[10]

Rearrangements of the *ret* proto-oncogene have only been found '*in vivo*' in thyroid gland tumours of the papillary histotype,[11–13] thus representing a fascinating example of organ and tumour specificity.

Despite the huge amount of information accumulated on *ret* activation in PTC oncogenesis, the clinical implications remain controversial. Some groups have reported a higher prevalence of *ret* activation in young patients,[14] whereas others have found gender to be a more important factor than age.[15] Viglietto *et al.*[16] and Santoro *et al.*[13] reported a higher frequency of *ret* activation in papillary microcarcinomas than in clinically evident PTCs, whereas most reports do not address this point. Finally, Mayr *et al.*[17] suggested that *ret* activation does not confer an increased risk of metastatic spread, in contrast to the data reported by Jhiang *et al.*[18] and Sugg *et al.*[19] The picture is even less clear concerning the putative relationship between *ret* activation and biological and/or molecular features of PTC, since most of the studies published to date either ignore this issue or address it using a non-systematic approach. Our aim was to address some of

*Correspondence to: Manuel Sobrinho-Simões, IPATIMUP, Medical Faculty of Porto, P-4200 Porto, Portugal.

Contract grant sponsor: JNICT; Contract grant number: PECS/C/SAU/254/95.

CCC 0123–3417/97/000000–00 $17.50

Received
Revised
Accepted

Table I—Comparison of the two groups of PTCs (with and without *ret* rearrangement) regarding the clinicopathological data

	Rearranged *ret* $n=8$	Non-rearranged *ret* $n=25$	*P* value
Gender (M:F)	1:7	1:3·2	NS
Mean age (years ± SE)	28·1 (± 3·1)	44·7 (± 2·9)	($P=0·005$)
Tumour diameter (cm ± SE)	3·3 (± 0·9)	3·3 (± 0·6)	NS
Tumour proliferative activity (% ± SE)	1·0 (± 0·2)	1·6 (± 0·2)	NS ($P=0·1$)
Histological subtype			
Predominantly papillary ($n=22$)	87·5%	60·0%	
Follicular variant ($n=11$)	12·5%	40·0%	NS ($P=0·15$)
Multicentricity	75·0%	59·1%	NS
Extrathyroidal growth	25·0%	24·0%	NS
Vascular invasion	25·0%	24·0%	NS
Lymphocytic infiltration			
Absent	28·6%	17·4%	
Discrete	71·4%	56·5%	
Moderate/abundant	0	26·1%	NS
Lymph node involvement*	62·5%	36·0%	NS ($P=0·19$)

*Only enlarged lymph nodes were removed and histologically examined. Fourteen of the 15 cases with enlarged lymph nodes had histologically confirmed metastatic deposits.

these controversies by comparing two groups of unselected PTCs one with and the other without *ret* rearrangement, with regard to several clinicopathological and biological features.

MATERIALS AND METHODS

Tumour specimens

Frozen specimens and paraffin blocks from 33 PTCs were retrieved from the files of the Unit of Molecular Pathology of IPATIMUP. The 33 tumours were surgically removed from patients consecutively operated upon in the Hospital S. João from April 1990 to April 1994 (Table I). The classification of the lesions was made according to Hedinger *et al.*[20] in sections stained with haematoxylin and eosin. There were 26 women and seven men. The mean age was 40·8 years (median 39 years; range: 13–77 years). Fifteen of the cases of PTC presented with enlarged lymph nodes and were therefore submitted to lymphadenectomy; in the remaining 19 cases, no involvement of the regional lymph nodes was noticed at surgery. The following pathological features were recorded in every case: size (cm), histological subtype, extrathyroidal growth, multicentricity (or intrathyroidal dissemination), vascular invasion, and lymphocytic infiltration (absent; scarce/moderate; abundant) (Table I). Eleven cases were exclusively or almost exclusively composed of follicles (follicular variant of PTC), whereas the remaining 22 cases displayed either a pure papillary or a mixed papillary–follicular pattern of growth. Follow-up information was obtained in every case. Recurrences were observed in two patients.

Southern blot analysis

High molecular weight DNA was isolated from frozen samples of the 33 primary tumours and two lymph node metastases using a standard salt method,[21] after confirming the presence of neoplastic tissue in adjacent sections. DNA was digested with EcoRI or HindIII endonuclease. The DNA fragments were separated by 0·7 per cent agarose gel electrophoresis and transferred to nylon membranes by the Southern method. Filters were hybridized with ^{32}P-labelled DNA probe prepared using a random primer kit. Two probes were used: a 1·8 kb BamHI fragment of *ret*/PTC1[4] (probe 1), which recognizes in normal DNA two EcoRI bands of 9 and 6·3 kb corresponding to H4 and *ret* fragments, respectively, and a 1 kb BglII–BamHI fragment specific for the 6·3 kb EcoRI fragment of proto-*ret*[5] (probe 2). Probe 2 is able to detect the region within the *ret* gene where all the oncogenic rearrangements characterized to date have been found.[5,6] The filters were washed and exposed to X-ray films at −70°C. The probes did not identify gene polymorphisms with the endonuclease enzymes used in the present study, as ascertained by the study of 40 samples of blood donors (data not shown).

Immunohistochemistry

Paraffin-embedded specimens were used in the immunohistochemical study. Serial sections were immunostained with monoclonal antibodies for Ki-67, a human nuclear proliferation-associated antigen (MIB-1, Immunotech), E-cadherin (HECD-1, Zymed), c-erb-B2 (NCL-CB11, Novocastra), c-met (kindly provided by Dr M. Pratt), Bcl-2 (NCL-Bcl-2, Novocastra), and vimentin

(Clone V9, Dako). Immunostaining was performed using the avidin–biotin–peroxidase (ABC) complex as described by Holm *et al.*,[22] with an additional step for microwave antigen retrieval whenever appropriate. Sections (5 μm) were cut and mounted on gelatin-coated slides. Antigen retrieval was carried out by microwave treatment, 5 × 1·5 min at 750 W in a 0·1% detergent solution[23] for E-cadherin antibody, and with citrate buffer (pH 6·0) for Bcl-2 and MIB-1 antibodies. For c-met and vimentin antibodies, no pretreatment was required. After cooling (20 min at room temperature), the sections were immersed in 0·3 per cent hydrogen peroxide (H_2O_2) in methanol to block endogenous peroxidase activity. Non-specific staining was eliminated by 20 min incubation with normal rabbit serum. Excess normal serum was removed, replaced by the specific monoclonal antibodies, and incubated overnight at 4°C. After washing the slides, sections were incubated
with a 1:200 dilution of biotin-labelled secondary antibody followed by ABC complex (avidin, 1:100 and biotin-labelled peroxidase, 1:100) for 30 min each. Subsequently, sections were stained for 7 min with 0·05 per cent 3,3-diaminobenzidine tetrahydrochloride, 0·01 per cent H_2O_2 in 0·05 M Tris–HCl buffer (pH 7·6), counterstained with haematoxylin, dehydrated and mounted. Normal serum was diluted in 25 per cent bovine serum albumin (BSA) and Tris-buffered saline (TBS), and primary antibodies, biotinylated secondary antibodies, and ABC were diluted in 12·5 per cent BSA in TBS.

Previously tested positive controls were included in all series for all the antibodies used. As negative controls, immunoglobulins of the same subclasses and in the same protein concentrations as the primary specific antibodies were used.

With the exception of the MIB-1 study (see below), all the immunohistochemical results were evaluated semi-quantitatively. Staining was recorded separately using a semi-quantitative and subjective grading, considering both the intensity of staining and the proportion of cells showing an unequivocal positive reaction. Percentage of immunopositive cells: 0, 0 per cent; 1, <25 per cent; 2, 25–<50 per cent; 3, 50–75 per cent; 4, >75 per cent. Intensity of staining: 0 (0), absent; 1 (0/+), very faint stain; 2 (+), faint; 3 (++), moderate; and 4 (+++), strong staining. For each case and each antibody, we calculated a staining score by multiplying the value attributed to the percentage of stained cells by that of the staining intensity. Localization of the staining in the cytoplasm and/or in the membrane was also recorded whenever possible.

Evaluation of the percentage of immunoreactive cells for MIB-1 was made by counting 1000 tumour cells in random fields.

Statistical analysis

The results are expressed as a percentage or a mean (± SE). Normal thyroid was compared with papillary carcinoma and within PTC, cases with *ret* rearrangement were compared with those without *ret* rearrangement. For statistical analysis, the chi-square test with the Yates correction, paired and unpaired Student's *t*-tests, and the ANOVA test were used. Two values were considered significantly different when $P<0·05$.

RESULTS

Southern blot analysis

Eight out of the 33 papillary carcinomas (24·2 per cent) presented with *ret* rearrangement. The rearranged bands were evident with both probes. One of the two cases in which both the primary tumour and the lymph node metastasis were analysed displayed rearranged bands in the primary tumour as well as in the metastasis (case 29), whereas the other only displayed rearranged bands in the metastasis (case 11) (Fig. 1).

Clinicopathological features

The comparison of the two groups of PTCs (with and without *ret* rearrangement) is summarized in Table I. The mean age of the patients with tumours displaying *ret* rearrangement (28 ± 9 years; median 30 years) was significantly ($P=0·005$) lower than that of the patients harbouring cases that did not present rearrangement (45 ± 15 years; median 44 years). No significant differences were found regarding the gender of the patients, tumour size, multicentricity, extrathyroidal growth, vascular invasion, lymphocytic infiltration, or lymph node involvement. There was a trend ($P=0·15$) towards a relationship between the presence of *ret* rearrangement and papillary or mixed papillary–follicular architecture: *ret* rearrangement was found in only one out of the 11 cases (9·1 per cent) of the follicular variant of PTC (Table I).

The tumours of the two patients who presented with recurrence of the disease (cases 4 and 20) were negative for *ret* rearrangement (Table II); no patient developed clinically evident metastases or died from the disease, but the follow-up period was too short to allow any meaningful prognostic conclusions to be drawn.

Immunohistochemical features

Some of the immunohistochemical results are summarized in Table II. The comparison of the immunohistochemical scores obtained in the PTC cases with those obtained in the respective adjacent normal thyroids is summarized in Table III. PTC displayed significantly higher expression of cytoplasmic E-cadherin, c-met, and vimentin, whereas the normal thyroid displayed significantly higher expression of Bcl-2. No significant differences were observed in the expression of E-cadherin (membrane) or c-erb-B2 (Table III).

The comparison of *ret*-rearranged PTCs and PTCs without *ret* rearrangement did not yield significant differences, apart from the higher expression of cytoplasmic E-cadherin in cases without *ret* rearrangement (Table IV).

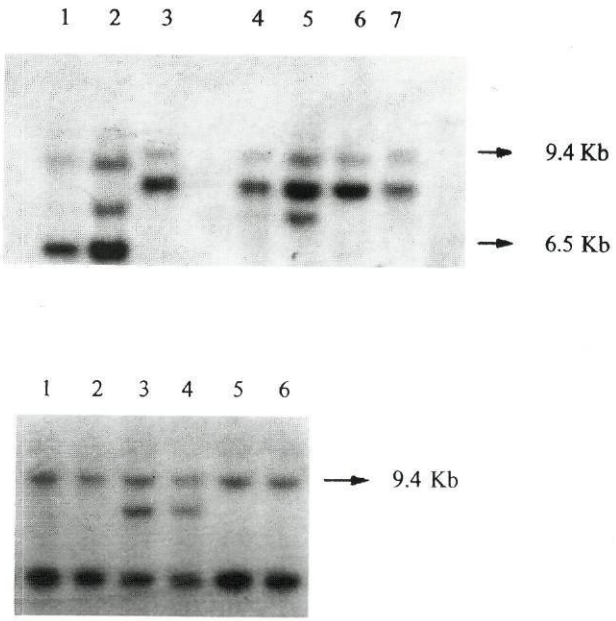


Fig. 1—Southern blot analysis of *ret* rearrangement in papillary carcinomas and lymph node metastases. DNA samples were digested with EcoRI and HindIII and hybridized to probe 1 (see Materials and Methods). Top panel: case 11—lanes 1, 2 (EcoRI) and 4, 5 (HindIII). Primary tumour (lanes 1 and 4) and lymph node metastasis (lanes 2 and 5) showing a rearranged band in the lymph node metastasis. Cases 4 (lane 3) and 19 (lane 6)—HindIII—no rearranged band is seen in any of these cases. Bottom panel: case 20 (lane 1), case 21 (lane 2), case 29 (lane 3—primary tumour and lane 4—lymph node metastasis), case 23 (lane 5) and case 24 (lane 6)—EcoRI. Rearranged bands are only seen in lanes 3 and 4 (primary tumour and metastasis of case 29)

MIB-1—The PTCs of our series exhibited low proliferative indices as evaluated by MIB-1 (Table II). There was a trend to a lower proliferative index in cases with *ret* rearrangement than in cases without rearrangement, but the difference did not reach the threshold of statistical significance ($P=0{\cdot}09$) (Fig. 2).

E-cadherin—The immunoreactivity for E-cadherin in most PTCs was weak and clearly heterogeneous, i.e., positive areas co-existed side-by-side with negative areas. The polarized expression of E-cadherin seen in normal thyroid was partly lost in every PTC, thus leading to less impressive membrane staining and more intense cytoplasmic staining in the PTC. Among the tumours, E-cadherin cytoplasmic immunostaining was stronger, though not significantly ($P=0{\cdot}13$), in cases that did not exhibit *ret* rearrangement (Fig. 3). The tendency to less widespread and less intense cytoplasmic immunoreactivity for E-cadherin in cases with *ret* rearrangement was reflected in the significantly ($P=0{\cdot}007$) lower score obtained in these cases in comparison with those without *ret* rearrangement (Table IV).

c-erb-B2—Cytoplasmic immunoreactivity for c-erb-B2 was observed in every PTC (Table II) as well as in the normal thyroid (Table III). Clear-cut membrane immunoreactivity for c-erb-B2 was not observed in any case.

c-met—The majority of the tumours presented cytoplasmic c-met (over)expression in contrast to the adjacent thyroid (Fig. 3). Eighty per cent of the papillary carcinomas of our series displayed immunoreactivity for this receptor (Table II). No significant difference was found between cases with and without rearrangement, regarding the percentage of immunoreactive cells or the intensity of staining (Table IV).

Bcl-2—Immunoreactivity for Bcl-2 was observed in most PTCs (Table II) as well as in the normal adjacent thyroid. The immunoreactivity was cytoplasmic and usually less intense in the carcinomas than in normal thyroid (Table III). No significant differences were found between cases with and without rearrangement,

Table II—Summary of the immunohistochemical results*

Case No.	Lesion*	Gender	Age (years)	*ret* rearr.	MIB-1 %	E-cad membrane	E-cad cytoplasm	c-erb-B2	c-met	Bcl-2
1	PT	F	38	NEG	1·1	ND	ND	+	ND	+++
2	PT	F	31	NEG	1·6	+	0/+	+	++	+
3	PT	F	26	NEG	0·2	ND	ND	+	+++	0/+
4	PT	F	44	NEG	0·8	++	0	++	+	++
5	PT	M	16	POS	1·2	ND	ND	++	ND	0
6	PT	F	49	NEG	ND	ND	ND	ND	ND	ND
7	PT	M	42	NEG	1·6	0/+	+	0/+	+++	+
8	PT	F	40	NEG	2·2	0/+	++	0/+	++	0/+
9	PT	M	49	NEG	3·0	ND	ND	+	ND	++
10	PT	M	72	NEG	2·2	ND	ND	+	ND	0
11	PT	F	37	NEG	0·2	+++	0/+	++	0	++
11	Met	F	37	POS	1·1	0/+	0/+	+	0/+	+
12	PT	F	44	NEG	1·0	ND	ND	+	ND	++
13	PT	F	53	NEG	1·3	ND	ND	++	ND	++
14	PT	F	31	POS	0·6	++	0/+	0/+	0	+++
15	PT	F	68	NEG	4·5	+	++	0/+	+	++
16	PT	F	51	NEG	1·2	++	++	+	++	+
17	PT	F	23	NEG	3·5	++	+	++	+++	0
18	PT	F	27	NEG	2·9	+	+	++	++	0
19	PT	M	57	NEG	1·0	ND	ND	+	ND	+
20	PT	M	77	NEG	0·8	++	++	++	+++	+
21	PT	F	33	NEG	1·0	+++	++	++	+	++
22	PT	F	36	POS	1·2	+	0	++	++	0/+
23	PT	F	22	NEG	2·1	+++	++	0/+	ND	+
24	PT	F	45	NEG	0·9	+++	+++	0/+	0/+	++
25	PT	F	47	NEG	2·2	0/+	+++	+++	++	++
26	PT	F	30	POS	0·4	ND	ND	+	ND	0/+
27	PT	M	30	NEG	1·6	0	0/+	+	++	+++
28	PT	F	13	POS	2·1	0/+	+	+++	+++	+
29	PT	F	32	POS	0·9	++	0/+	++	+	0
29	Met	F	32	POS	ND	ND	ND	ND	ND	ND
30	PT	F	60	NEG	0·4	0/+	++	++	+++	+++
31	PT	F	30	POS	0·3	0/+	0/+	+++	+++	++
32	PT	F	41	NEG	1·5	++	+	++	++	+
33	PT	F	58	NEG	1·3	+	++	+	+++	0/+

*PT=primary tumour; Met=lymph node metastasis.
ND=not done.

Table III—Summary of the immunohistochemical scores in PTC and in normal thyroid

Immunohistochemical score	Normal thyroid	PTC	*P* value*
E-cadherin—membrane (SE)	8·1 (0·9)	6·6 (0·9)	NS
E-cadherin—cytoplasm (SE)	2·6 (0·3)	6·0 (0·8)	0·002
c-erb-B2 (SE)	9·4 (0·6)	7·6 (0·8)	NS
c-met (SE)	0·0 (0·0)	10·3 (1·1)	<0·0001
Bcl-2 (SE)	11·1 (0·4)	5·6 (0·9)	0·0001
Vimentin (SE)	0·0 (0·0)	7·0 (1·0)	<0·0001

*Similar results were obtained with the paired and unpaired Student's *t*-test.

regarding the percentage of immunoreactive cells or the intensity of staining (Table IV).

Vimentin—Almost every PTC displayed immunoreactivity for vimentin, in contrast to the adjacent normal thyroid (Fig. 3). The expression of vimentin was localized in the basal poles of the cancer cells in the large majority of PTCs, regardless of the presence or absence of *ret* rearrangement. The two groups of PTCs did not differ in relation to the percentage of immunoreactive cells and the intensity of staining (Table IV).

Table IV—Comparison of the immunohistochemical scores in PTCs with and without *ret* rearrangement*

Immunohistochemical score	Rearranged *ret*	Non-rearranged *ret*	*P* value
E-cadherin—cytoplasm (± SE)	2·7 (0·7)	8·0 (1·1)	0·007
c-met (± SE)	8·8 (2·6)	10·9 (1·2)	NS
Bcl-2 (± SE)	3·6 (1·9)	6·2 (1·1)	NS
Vimentin (± SE)	7·3 (1·7	6·6 (1·1)	NS

*We have included only those markers in which PTCs differed significantly from normal thyroid (Table III).

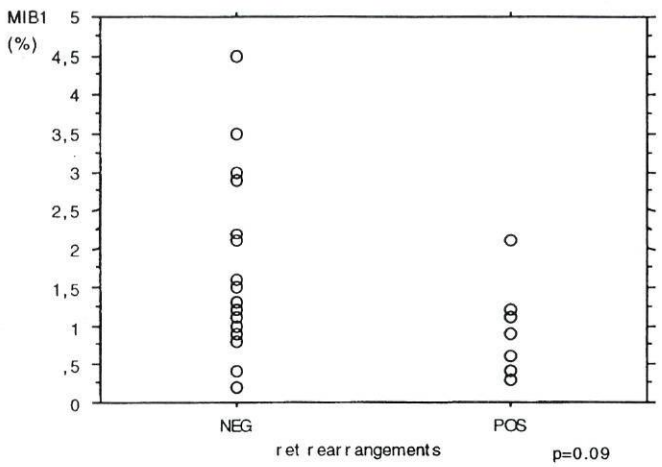


Fig. 2—Scattergram of the percentage of MIB-1 immunoreactive cells in cases with (n=8) and without (n=24) *ret* rearrangement. *In one case, MIB-1 immunoreactivity was not evaluated (for technical reasons)

DISCUSSION

About one in four of the cases of the present series displayed *ret* rearrangement. The 24·2 per cent prevalence that we have detected is within the range previously reported in western European countries[3,13,24] and contrasts with the low prevalence of *ret* rearrangement reported in Japanese, Arabian, and Canadian series.[19,25,26]

Our finding of a significantly lower age in patients with tumours displaying *ret* rearrangement fits with the results of Bongarzone *et al.*,[14] Jhiang *et al.*,[18] and Sugg *et al.*,[19] and supports the association of *ret* rearrangement and early onset of PTC. This association is further supported by the concurrent increase in the incidence of thyroid carcinoma in children after the Chernobyl disaster[27] and by the high frequency of *ret* activation in these cases (about 60 per cent).[8,9,28] The association between *ret* rearrangement and early onset of PTC may reflect the existence of a short-cut (single or few events) in *ret*-induced papillary carcinogenesis and/or that the functional status of the thyrocytes of youngsters makes them a more sensitive target for *ret*-activated signal, as suggested by Bongarzone *et al.*[14]

The *in vitro* experiments of Bond *et al.*[29] using amphotropic vectors indicated that *ret* rearrangement has the potential to act as an initiator event of papillary tumours, in contrast to *ras*, which seems to be an initiating event in the follicular histotype. This possibility is supported by experiments using transgenic mice with targeted expression of *ret*/PTC1 oncogene, which developed thyroid tumours that were considerably similar to human papillary carcinomas.[30] The finding of Viglietto *et al.*[16] and Santoro *et al.*[13] of a much higher frequency of *ret* rearrangements in papillary microcarcinomas than in clinically evident PTCs has been advanced as an extra argument favouring the association between *ret* rearrangement and initiation of PTC.[16]

It remains to be determined whether most microcarcinomas are indeed recently developed PTCs, or relatively 'old' lesions that did not develop further or are even regressing. The scarred appearance of many microcarcinomas is in keeping with the latter possibility.[31,32] It is even possible that some of these tiny carcinomas may actually regress completely.

The high prevalence of *ret* rearrangement detected in papillary microcarcinomas by Viglietto *et al.*[16] and

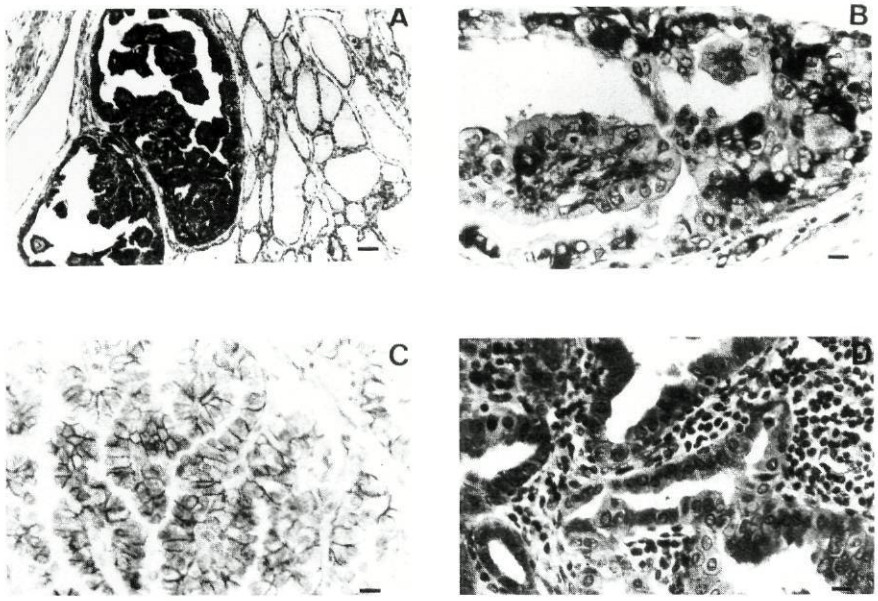


Fig. 3—Examples of immunoreactivity in papillary carcinomas for some of the markers used in this study. (A) c-met expression in a focus of papillary carcinoma. Note the contrast between the neoplastic cells and the adjacent normal parenchyma (anti-c-met; scale bar = 60 μm). (B) Prominent vimentin expression is observed in the cytoplasm mainly in the basal poles of some of the neoplastic cells (anti-vimentin; scale bar = 15 μm). (C) Heterogeneous E-cadherin membrane expression is observed in this focus of papillary carcinoma (anti-E-cadherin; scale bar = 15 μm). (D) Strong E-cadherin expression in the cytoplasm of the neoplastic cells (anti-E-cadherin; scale bar = 15 μm)

Santoro *et al.*[13] supports the hypothesis that tumours harbouring a *ret*/PTC activation could proceed less frequently towards large PTCs than papillary tumours lacking *ret* rearrangement.[16] The similarity of the size of the PTC with and without *ret* rearrangement observed in our series does not clarify this issue, because our cases were clinically selected and thus represent a biased sample.

The trend to a lower proliferative rate in *ret*-rearranged PTCs detected in the present study fits with the reported association between papillary microcarcinomas and *ret* rearrangement.[13,16] This low proliferative potential also fits with the findings of Bond *et al.*[29] of a poorer growth potential *in vitro* of thyrocytes transformed with amphotropic vectors carrying activated *ret* when compared, for example, with *ras*-activated cells. It remains to be determined whether the apparently greater clinical aggressiveness of the so-called post-Chernobyl PTC[27] reflects the influence of other types of *ret* rearrangement,[8,9] or the co-existence in this setting of other contributory factors, such as the higher normal proliferation rate of follicular cells in children.

Other types of evidence reinforce the assumption that most *ret*-rearranged PTCs do not tend to progress towards more advanced steps of neoplastic development. Several groups did not find *ret* rearrangemnets in less-differentiated thyroid tumours, despite the frequent occurrence of loci of PTCs in poorly differentiated and undifferentiated carcinomas.[11,13] This suggests that tumours harbouring *ret* rearrangements do not usually evolve to undifferentiated phenotypes,[13] or if they do, they lose the rearrangement during the process. Conversely, mutation of p53 has been detected to date, to the best of our knowledge, in only one PTC displaying *ret* rearrangement.[26] Most authors did not find p53 inactivation in PTCs with or without *ret* rearrangement, even when foci of p53 immunoreactive undifferentiated carcinoma co-existed with PTC.[33–35] Finally, it has been observed that the introduction of mutant p53 by means of an amphotropic retroviral vector has no effect on human thyroid cells harbouring *ret* rearrangement (Wyllie FS, personal communication).

Curiously, the apparently restricted tendency of *ret*-rearranged PTCs to evolve to less-differentiated phenotypes does not interfere with their nodal metastatic ability. In fact, series on record show, like our own, and *ret*-rearranged PTCs usually display a predisposition to lymphatic involvement.[18,19] As has been stressed by Sugg *et al.*,[19] it is still not known why *ret*-rearranged PTCs give rise to lymph node metastases despite often occurring as small, slow growing, quite harmless tumours.

The search for immunohistochemically detectable differences between PTCs with and without *ret* rearrangement yielded very disappointing results. In fact, and at variance with the clear-cut distinction between normal

thyroid and PTC, no major features could be ascribed to PTCs exhibiting *ret* rearrangement, apart from lower proliferative activity. The patterns of immunostaining of E-cadherin and c-erb-B2, as well as those of c-met and vimentin, fit with previously reported data on the immunohistochemical expression of these antigens in PTC[23,36,37] and does not throw any light on the pathogenesis of *ret*-rearranged PTC. The series is, however, too small to allow a meaningful interpretation of the results obtained in the two subsets of PTCs.

ret rearrangements are usually found both in the primary tumours and in the corresponding lymph node metastases.[3,5] One case of our series showed *ret* activation only in the lymph node metastasis. This patient was the eldest of the patients with tumours displaying *ret* rearrangement, thus suggesting that *ret* rearrangement may occur as a secondary event during the course of the disease. However, one cannot rule out that the absence of detectable *ret* rearrangement in the primary tumour may result from the contamination of neoplastic cells by stromal and normal thyroid cells, preventing the detection of the extra bands.

Taken together, our results and most published studies are consistent with a lack of 'potency' of *ret* in comparison with most activated oncogenes. This would explain the failure of most papillary microcarcinomas to develop further and, if they do, their failure to go on to undifferentiated carcinomas. As a consequence, PTCs harbouring a *ret* rearrangement occur frequently as tiny, slow growing, predominantly papillary tumours that do not usually progress towards more aggressive neoplasms, despite giving rise to regional lymph node metastases. They seem to represent a sort of Bonsai phenomenon in tumour biology.

ACKNOWLEDGEMENTS

We thank Miss Paula Silva for her help in the immunohistochemical study and Dr Maria Pratt for providing c-met antibody. This work was supported by JNICT (grant No. PECS/C/SAU/254/95).

REFERENCES

1. Hay ID. Papillary thyroid carcinoma. *Endocrinol Metab Clin North Am* 1990; **19**: 545–576.
2. Sobrinho-Simões M. Tumours of the thyroid. A brief overview with emphasis in the most controversial issues. *Curr Diagn Pathol* 1995; **2**: 15–22.
3. Fusco A, Grieco M, Santoro M, *et al.* A new oncogene in human thyroid PTCs and their lymph node metastases. *Nature* 1987; **328**: 170–172.
4. Grieco M, Santoro M, Berlingieri MT, *et al.* PTC is a novel rearranged form of the ret proto-oncogene and is frequently detected *in vivo* in human thryoid papillary carcinomas. *Cell* 1990; **60**: 557–563.
5. Bongarzone I, Butti MG, Coronelli S, *et al.* Frequent activation of *ret* protooncogene by fusion with a new activating gene in papillary thyroid carcinomas. *Cancer Res* 1994; **54**: 2979–2985.
6. Sozzi G, Bongarzone I, Miozzo M, *et al.* A t(10;17) translocation creates the RET/PTC2 chimeric transforming sequence in papillary thyroid carcinoma. *Genes Chrom Cancer* 1994; **9**: 244–250.
7. Jhiang SM, Smanik P, Mazzaferri E. Development of a single-step duplex rt-PCR detecting different forms of the third form of *in vivo ret* activation in human papillary thyroid carcinoma. *Cancer Lett* 1994; **78**: 69–76.
8. Fugazzola L, Pierotti M, Vigano E, Pacini F, Vorontsova T, Bongarzone I. Molecular and biochemical analysis of RET/PTC4, a novel oncogenic rearrangement between RET and ELE1 genes, in a post-Chernobyl papillary thyroid cancer. *Oncogene* 1996; **13**: 1093–1097.
9. Klugbauer S, Lengfelder E, Demidchik EP, Rabes HM. A new form of ret rearrangement in thyroid carcinomas of children after the Chernobyl reactor accident. *Oncogene* 1996; **13**: 1099–1102.
10. Trupp M, Arenas E, Fainzilber M, *et al.* Functional receptor for GDNF encoded by the c-ret proto-oncogene. *Nature* 1996; **381**: 785–789.
11. Santoro M, Carlomagno F, Hay ID, *et al.* RET oncogene activation in human thyroid neoplasms is restricted to the papillary cancer subtype. *J Clin Invest* 1992; **89**: 1517–1522.
12. Santoro M, Sabino N, Ishizaka Y, *et al.* Involvement of ret oncogene in human tumours: specificity of ret activation to thyroid tumours. *Br J Cancer* 1993; **68**: 460–464.
13. Santoro M, Tallini G, Carlomagno F, *et al.* Activation of the RET proto-oncogene in human thyroid tumours. *J Endocrinol Invest* 1996; **19** (Suppl to No 6): 4..
14. Bongarzone I, Fugazzola L, Vigneri P, *et al.* Age-related activation of the tyrosine kinase receptor proto-oncogenes ret and ntrk1 in papillary thyroid carcinoma. *J Clin Endocrinol* 1996; **81**: 2006–2009.
15. Williams GH, Thomas GA, Voscoboinik L, *et al.* Rearrangements of the RET oncogene in papillary carcinoma and gender. *J Endocrinol Invest* 1996; **19** (Suppl to No 6): 6..
16. Viglietto G, Chiappetta G, Martinez-Tello F, *et al.* RET/PTC oncogene activation is an early event in thyroid carcinogenesis. *Oncogene* 1995; **11**: 1207–1210.
17. Mayr B, Pötter E, Goretzki P, *et al.* Prognostic value of ret-proto-oncogene activation in papillary thyroid carcinoma. *J Endocrinol Invest* 1996; **19** (Suppl to No 6): 57..
18. Jhiang SM, Caruso DR, Gilmore E, *et al.* Detection of the ptc/ret^{PTC} oncogene in human thyroid cancers. *Oncogene* 1992; **7**: 1331–1337.
19. Sugg SL, Zheng L, Rosen IB, Freeman JL, Ezzat S, Asa SL. *ret*/PTC-1, -2 and -3 oncogene rearrangement in human thyroid carcinomas: implications for metastatic potential? *J Clin Endocrinol Metab* 1996; **81**: 3360–3365.
20. Hedinger C, Williams ED, Sobin LH, (eds). Histologic Typing of Thyroid Tumours. 2nd edn. World Health Organization—International Histologic Classification of Tumors. Berlin: Springer-Verlag, 1988.
21. Mullenbach R, Weldar C, Lagoda P. An efficient salt chloroform extraction of DNA from blood and tissues. *Trends Genet* 1989; **5**: 391..
22. Holm R, Sobrinho-Simões M, Nesland JM, Gould VE, Johannessen V. Medullary carcinoma of the thyroid gland. An immunohistochemical study. *Ultrastruct Pathol* 1985; **8**: 25–41.
23. Soares P, Berx G, Van Roy F, Sobrinho-Simões M. E-cadherin gene alterations are rare events in thyroid tumors. *Int J Cancer* 1997; **70**: 32–38.
24. Bongarzone I, Pierotti MA, Monzini N, *et al.* High frequency of activation of tyrosine kinase oncogenes in human papillary thyroid carcinoma. *Oncogene* 1989; **4**: 1457–1462.
25. Wajjwalku W, Nakamura S, Hasegawa Y, *et al.* Low frequency of rearrangements of the *ret* and *trk* proto-oncogenes in Japanese thyroid PTCs. *Jpn J Cancer Res* 1992; **83**: 671–675.
26. Zou M, Shi Y, Fadir N. Low rate of *ret* proto-oncogene activation (PTC/*ret*^{PTC}) in papillary thyroid carcinomas from Saudi Arabia. *Cancer* 1994; **73**: 176–180.
27. Nikiforov Y, Gnepp DR. Pediatric thyroid cancer after the Chernobyl disaster. *Cancer* 1994; **74**: 748–766.
28. Klugbauer S, Lengfelder E, Demidchik EP, Rabes HM. High prevalence of ret rearrangement in thyroid tumors of children from Belarus after the Chernobyl reactor accident. *Oncogene* 1995; **11**: 2459–2467.
29. Bond JA, Wyllie FS, Rowson J, Radulescu A, Wynford-Thomas D. *In vitro* reconstruction of tumour initiation in a human epithelium. *Oncogene* 1994; **9**: 281–290.
30. Jhiang SM, Sagartz JE, Tong Q, *et al.* Targeted expression of the ret/ptc1 oncogene induces papillary thyroid carcinomas. *Endocrinology* 1996; **137**: 375–378.
31. Woolner LB, Lemmon ML, Bearhs OH, Black BM, Keating FR. Occult papillary carcinoma of the thyroid gland—a study of 140 cases observed in a 30-year period. *J Clin Endocrinol Metab* 1960; **20**: 89–105.
32. Sobrinho-Simões M, Sambade MC, Gonçalves V. Latent thyroid carcinoma at autopsy. A study from Oporto, Portugal. *Cancer* 1979; **43**: 1702–1706.
33. Fagin JA, Matsuo K, Karmakar A, Chen DL, Tang SH, Koeffler HP. High prevalence of mutation of p53 gene in poorly differentiated human thyroid carcinomas. *J Clin Invest* 1993; **91**: 179–184.
34. Matias-Guiu X, Cuatrecasas M, Musulen E, Prat J. p53 expression in anaplastic carcinoma arising from thyroid papillary carcinomas. *J Clin Pathol* 1994; **47**: 377–339.
35. Williams ED. Pathology and molecular biology of post Chernobyl thyroid carcinoma. *J Endocrinol Invest* 1996; **19** (Suppl to No 6): 4..
36. Di Renzo MF, Olivero M, Ferro S, *et al.* Overexpression of the c-MET/HGF receptor gene in human thryoid carcinomas. *Oncogene* 1992; **7**: 2549–2553.
37. Soares P, Sambade C, Sobrinho-Simões M. Expression of c-erb B2 in tumours and tumour-like lesions of the thyroid. *Int J Cancer* 1994; **56**: 459–461.

Solicitou-se ao PRAXIS XXI um subsídio para custear parte das despesas com a publicação da presente dissertação.

EXECUÇÃO GRÁFICA

GRÁFICA VISAGUE, LDA.
RUA DA ALEGRIA, 315 - 4000 PORTO
TEL. 02 3320955 - FAX - 02 3325746