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Molecular Biology of Thyroid Cancer

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1. Introduction

Thyroid is a H-shaped gland localised in front of trachea at the base of the neck, whose main functions are the synthesis, the storage and the secretion of thyroid hormones under the control of the hypothalamic–pituitary axis. Thyroid is comprised of spherical follicles filled with colloid that are lined by cuboidal/flat epithelial cells denoted follicular cells (or thyrocytes). The other hormone-producing cells in the thyroid gland are scattered within follicles, and are denoted para-follicular cells (or C cells). Whereas follicular cells are responsible for iodine uptake and thyroid hormone synthesis, C cells are dedicated to the production of calcitonin (Dumont et al., 1992).

Cancers that arise in the thyroid gland represent the most common malignancy of the endocrine system and accounts for approximately 1% of all newly diagnosed cancer cases in Western countries, with estimates of annual incidence rates of 12 cases per 100,000 in North America and 5.6 new cases per 100,000 in Europe (Gilliland et al., 2009). Incidence rates of thyroid cancer widely vary worldwide, possibly because of inherent ethnic geographical or environmental differences that include iodine deficiency and radiation exposure. For instance the incidence of thyroid cancer is high in the Chinese and Filipino population of Hawaii (119 cases/million women and 45 cases/million men, respectively) and it is relatively low in Poland (14 cases/million women and 4 cases/million men, respectively) (Ain, 1995). The most common forms of thyroid carcinoma derive either from thyroid follicular epithelial cells or from C cells (Sherman, 2003). The former include well-differentiated carcinoma (WDTC) - divided into (PTC) and follicular thyroid carcinoma (FTC) -, poorly differentiated carcinoma (PDTC) and anaplastic thyroid carcinoma (ATC) (Rosai et al., 1992; DeLellis et al., 2004). PTC is the most frequent type of thyroid malignancy, and accounts for approximately 80-85% of all cases, FTC accounts for approximately 10-15% of all thyroid tumors whereas PDTC and ATC are rare aggressive malignancies (<2% of all thyroid cancer) that can develop either directly or from pre-existing well-differentiated PTC and FTC. Thyroid cancer derived from para-follicular C cells is denoted Medullary Thyroid Carcinoma (MTC). MTC is a relatively rare malignancy (<5%) and will not be discussed here. Most neoplasms derived from thyroid follicular epithelial cells are indolent tumours that can be effectively treated by surgical resection and/or radioactive-iodine administration. Usually, PTC and FTC are well-differentiated tumours with a fairly good prognosis that are generally curable with current treatments (Sherman,

2003). By contrast, PDTC and ATC represent partially or completely undifferentiated form of thyroid cancer that behave aggressively, and for which there is currently no effective treatment. Accordingly, patients with PDTC or ATC have a mean life expectancy of few months, representing the major therapeutic challenge for thyroid cancer therapy (Cornett et al., 2007). A study of nearly 16,000 patients in the United States estimated the survival rates for the various types of thyroid cancer to be 98% for PTC, 92% for FTC, and 13% for ATC. The main cause of thyroid cancer-related mortality is due to the surgical inoperability at diagnosis of many patients and to the frequent insensitivity exhibited by advanced thyroid cancer patients to radioiodine treatment. Therefore, there is the need for ameliorating the comprehension of thyroid tumorigenesis and for improving the treatment of patients with PDTC and ATC. This Chapter will focus on the mechanisms that underlie onset and progression of the more common neoplasms that originate from thyroid follicular cells and on novel targeted therapeutic strategies developed to treat thyroid cancer patients.

1.1 Epidemiology and risk factors of thyroid cancer

The main risk factors identified so far that contribute to the development of thyroid carcinoma are radiation exposure, reduced iodine intake, thyroiditis, hormonal factors and family history. Radiation exposure, especially if during infancy, represents the most important risk factor for PTC development, as demonstrated by several studies on the consequences of the explosions of atomic bombs of Hiroshima and Nagasaki (1945), nuclear testing in the Marshall Islands (1954) and Nevada (1951–1962), and of the more recent nuclear accident in Chernobyl (1986). Exposure to internal sources of ^{131}I as after the Chernobyl nuclear accident has led to a 3- to 75-fold increase in the incidence of PTC, with the highest effects most pronounced in children (Cardis et al., 2005). Similarly, exposure to external beam-radiation delivered between 1920 and 1950 for the treatment of benign conditions of the head and neck - such as thymic enlargement, tonsillitis, acne, and adenitis - and currently for Hodgkin's lymphoma, also has increased the risk of PTC of 3 to 9 fold per Gy. As suggested above, radiation exposure during childhood is more likely to produce thyroid neoplasia than similar exposure at a later age, because of the greater cellular mitotic activity shown by thyrocytes in the young. There is a linear relationship between radiation doses and the incidence of thyroid nodules and cancer. Most nodules tend to occur within 10 to 20 years of exposure, but the risk for development of malignant nodules may exist for over 40 years. The typical molecular lesion induced by radiation seems to be the chromosomal rearrangement as opposed to point mutation as a mode of aberrant gene activation associated to iodine deficiency (Ron et al., 1995).

A second risk factor for well-differentiated thyroid carcinoma is iodine deficiency (Sherman, 2003). Dietary iodine deficiency results in thyroid proliferation as a compensatory mechanism, which is the likely cause of goiter development. Interestingly, the incidence of FTC is higher in areas of iodine deficiency whereas PTC is the most frequent type of thyroid cancer in iodine-sufficient regions. However, the role of iodine in thyroid carcinogenesis is still unclear. Studies in experimental thyroid cancer systems have suggested that the role of iodine in thyroid carcinogenesis can be to modulate tumour morphology, causing the change from follicular to papillary morphology, more than decreasing overall tumor incidence (Yamashita et al., 1990). Another recognised risk factor that might predispose to the development of thyroid malignancies is the presence of some underlying inflammatory thyroid diseases (i.e. thyroiditis). Indeed, about a third of patients affected by thyroid

carcinoma present benign thyroid disease such as Hashimoto's disease, multinodular or adenomatoid goiter. Moreover, the finding that PTC frequently contains lymphocytic infiltration indicates that immunological factors might be involved in the initiation and/or progression of thyroid carcinoma. Recent studies have identified precursor lesions embedded inside chronic lymphocytic thyroiditis, though it remains to be determined whether this represents a reactive response or a prerequisite for tumorigenesis (Gasbarri et al., 2004). Thyroid cancer presents a marked sex- and age-specific incidence, being 2–4 times more frequent in females than in males (Gilliland et al, 2009; Sherman, 2003). This suggests that female hormones might regulate thyroid carcinogenesis. However, although it has been shown that oestrogen promotes the proliferation of thyrocytes there is no clear causal relationship between thyroid cancer and pregnancy or the use of exogenous sex hormones. Finally, the existence of a genetic component that may predispose to development of thyroid cancer has been also suggested. Family history with a parent or a sibling affected by follicular cell-derived thyroid carcinoma increases risk 3.2- and 6.2-fold, respectively (Hemminki et al., 2005). Putative susceptibility loci have been identified on chromosomes 1q21, 2q21, and 19p13.2.21. Other thyroid cancer susceptibility loci have been identified in familial tumour syndromes that predispose to PTC in association with papillary renal cell carcinoma (1q21), clear-cell renal-cell carcinoma ((3;8)(p14.2;q24.1)), and multinodular goiter (19p13.2) (Eng, 2000). Finally, familial thyroid cancers have been associated with inherited tumour syndromes that include familial Polyposis coli and the related Gardner and Turcot syndromes (associated with mutations in the adenomatosis polyposis coli gene (APC)), Cowden disease (associated with mutations in the phosphatase with tensin homology gene (PTEN)), Werner syndrome (associated with mutations in the WRN gene) and Carney complex (associated with mutations in the PRKAR1A gene, encoding the type 1A regulatory subunit of protein kinase) (Lindor & Greene, 2008).

1.2 Molecular pathogenesis of thyroid cancer

Tumors originating from thyroid follicular cells provide an excellent model to understand the development of human cancer. Thyroid nodules can be either benign tumors (hyperplastic goiter, adenoma) or malignant cancers. Knowledge of the molecular events that govern human thyroid tumorigenesis has grown considerably in the past twenty years leading to the identification of key genetic alterations and new oncogenic pathways implicated in cancer initiation and/or development (Nikiforova & Nikiforov 2008; Xing, 2008). In addition, it has become apparent that distinct molecular events are associated with specific stages of the multistep tumorigenic process, with a good genotype/phenotype correlation. In this section we will briefly review the pathological features of thyroid benign and malignant tumors, describing the molecular alterations identified so far.

1.2.1 Benign tumors

Goiter is an enlargement of the thyroid gland that is caused either by a primary thyroid disease or by aberrant stimulation of the gland due to an excess of blood hormone levels, autoantibodies or other factors. Thyroid adenomas represent benign epithelial tumours in which the cells are derived from the follicular epithelium and form recognizable follicular structures composed mostly of terminally differentiated thyrocytes (Figure 1). At the molecular levels, benign hyperfunctioning thyroid nodules as well as thyroid adenomas have been associated with activating mutations in the gene encoding the thyroid-

stimulating hormone receptor (TSHR) or the GNAS1 gene encoding the GSα subunit of the TSHR-coupled guanine nucleotide-binding proteins (G-proteins). Both mutations constitutively activate the adenylyl cyclase–cyclic AMP (cAMP) cascade thereby regulating the growth of follicular cells (Krohn et al., 2005).
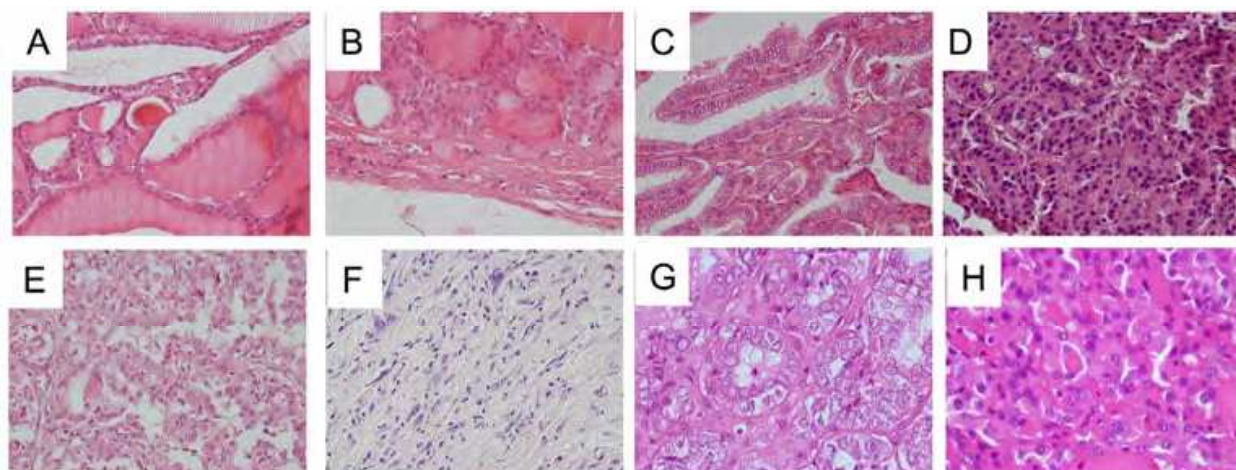

Fig. 1. Different histotypes of human thyroid cancer. A, Normal Thyroid. B, Adenoma. C, Classical Papillary Thyroid Carcinoma. D, Follicular Thyroid Carcinoma. E, Poorly Differentiated Thyroid Carcinoma. F, Anaplastic Thyroid Carcinoma; G. Typical PTC characterized by the presence of papillae, crowded nuclei with grooves and "ground glass" appearance. H, Hurthle-cell Thyroid Carcinoma. Courtesy of Dr. Renato Franco (INT Fondazione Pascale, Napoli, Italy).

1.2.2 Malignant cancers

Well-differentiated thyroid carcinomas are composed of differentiated follicular epithelial cells. Most well-differentiated thyroid cancers behave in an indolent manner and have an excellent prognosis. There are two main groups, PTC and FTC, each of which has several variants. PTC might occur in several histologic subtypes including classical form with papillary architecture, follicular variant, oncocytic variant (or Hurthle-cell variant), tall-cell variant or solid and cribriform types, each showing distinct patterns of growth and clinical behaviours (Rosai et al., 1992; DeLellis et al., 2004). The classical form of PTC is the most common and is a relative indolent disease with good prognosis. It is characterized by distinctive features such as the presence of papillae (consisting of a well-defined fibrovascular core surrounded by one or two layers of tumor cells), crowded nuclei with grooves and "ground glass" appearance, cytoplasmic pseudoinclusions caused by a redundant nuclear membrane, and Psammoma body (scarred and calcified remnants of infarcted papillae) (Figure 1C and G). Follicles and colloid are typically absent in PTC. The follicular variant accounts for approximately 10% of all PTC. It presents with cells organized into follicles rather than papillae, but at the cytological level, it displays the typical nuclear features of PTC. Overall survival and recurrence rates of follicular variant PTC are similar to those shown by the common type. By contrast, the tall-cell variant PTC is more aggressive, being characterized by cells with eosinophilic cytoplasm that are twice as tall as they are wide (Stojadinovic et al., 2001). In the tall-cell variant tumors tend to be large and invasive, and frequently patients present both local and distant metastases at the time of diagnosis.

The most studied pathway involved in PTC tumorigenesis is the RTK/RAS/BRAF/MAP kinase pathway, which is apparently essential for the development of PTC (Nikiforova & Nikiforov 2008; Xing, 2008). By contrast, this pathway seems to play a more limited role in FTC. At least three initiating events have been shown to occur in PTC: i) point mutations in the RAS genes; ii) point mutations in the BRAF gene; and iii) rearrangements of RET/PTC or neurotrophic tyrosine kinase receptor 1 (NTRK1) following radiation exposure (Nikiforova & Nikiforov 2008; Xing, 2008). The occurrence of mutually exclusive mutations of RET/PTC, TRK1, RAS or BRAF provides compelling genetic evidence for the critical role of the MAPK pathway in onset and/or progression of PTC. Unregulated activation of other tyrosine kinase receptors such as EGFR or MET may also represent a common step in the onset of PTC. See Table 1 for a summary of genetic alterations detected in thyroid cancer.

FTC is composed of well-differentiated follicular epithelial cells that lack the nuclear features of PTC that is characterized by haematogenous spread (Figure 1D). Typically, these tumors are encapsulated, and presents invasion along the capsule or across vascular endothelium (Rosai et al., 1992; DeLellis et al., 2004). Although cytologic features do not reliably allow discriminate between benign and malignant follicular lesions FTC may be distinguished from benign adenoma on the basis of the presence of invasive foci determined at the histological level. At difference with PTC, where the lack of a pre-malignant precursor has hindered the identification of the key steps in malignant transformation, it is generally hold that FTC may arise from benign thyroid adenoma as a result of transforming events. The two known initiating events in FTC are RAS mutations and the chromosomal translocation t(2;3)(q13;p25) that fuses the DNA binding domain of PAX8 to peroxisome proliferator-activated receptor (PPAR)γ (PAX8-PPARγ) (Nikiforova & Nikiforov 2008). Mutations in RAS, which are common in follicular adenomas, may lead to greater genomic instability, with increased allelic loss and more risk for transforming PAX8-PPARγ rearrangements that lead to development of FTC. Aberrant activation of the phosphatidylinositol-3 kinase (PI3K)/AKT pathway plays a fundamental role in FTC. Alterations within the PI3K/AKT pathway detected so far in thyroid tumors include mutations and genomic amplification/copy gain of the p110 catalytic subunit of PI3K (PIK3CA), PIK3CB, AKT1 and AKT2 and loss of PTEN through inactivating mutations, LOH or promoter methylation. Most of these genetic alterations are particularly common in FTC and in ATC but less common in PTC, in which the MAP kinase pathway, activated by the BRAF mutation or RET/PTC rearrangements, apparently plays a major role (Nikiforova & Nikiforov 2008; Xing, 2008). Many of these genetic alterations are mutually exclusive with increasing co-existence in ATC.

Variants of FTC include oncocytic (Hurthle-cell) and clear-cell types. Hurthle cell tumours are formed by cells containing numerous altered mitochondria, which confer the typical granular, eosinophilic appearance to their cytoplasm (Stojadinovic et al., 2001) (Figure 1H). Most Hurthle cell tumours have a follicular architecture and are diagnosed as adenoma or carcinoma on the basis of the same criteria applied to other follicular tumors - the identification of invasive behaviour. A Hurthle-cell variant of PTC also exists, though it is much less common than typical PTC. They present RET rearrangements and BRAF mutations and tends to be more aggressive than classical PTC (Cheung et al., 2000). Deletions and/or point mutations in mitochondrial DNA (mtDNA) are common in non-neoplastic and neoplastic thyroid cells that show morphological oncocytic changes (Yeh et al., 2000). However, although a role of mtDNA mutation in cell growth and tumorigenicity

TUMOUR TYPE	PREVALENCE	AGE (YEARS)	LYMPHNODE METASTASIS	DISTANT METASTASIS	SURVIVAL RATE (5 YEARS)	GENETIC ALTERATION
PTC	85-90%	20-50	< 50%	5-7%	> 90%	RET rearrangement (13-43%) BRAF mutation (29-69%) BRAF rearrangement (1%) NTRK1 rearrangement (5-13%) Ras mutation (0-21%) PIK3CA amplification (5-14%) PIK3CA mutation (0-3%) AKT1 amplification (n.d.) Akt1 mutation (n.d.)
FTC	< 10%	40-60	< 5%	20%	> 90%	Ras mutation (40-53%) PPARG rearrangement (25-63%) TP53 mutation (0-9%) PIK3CA amplification (24-28%) PIK3CA mutation (6-13%) AKT1 amplification (0-8.2%) AKT1 amplification (0-18.8%) Akt1 mutation (0%)
PDTC	0-7%	50-60	30-80%	30-80%	50%	RET rearrangement (0-13%) BRAF mutation (13-47%) Ras mutation (18-27%) CTNNB1 mutation (0-25%) TP53 mutation (17-38%) PIK3CA amplification (0-21%) PIK3CA mutation (5-21%) AKT1 mutation (0-16%)
ATC	2%	60-80	40%	20-50%	1-17%	BRAF mutation (10-35%) Ras mutation (20-60%) CTNNB1 mutation (66%) TP53 mutation (67-88%) PIK3CA amplification (0-42%) PIK3CA mutation (12-23%) AKT1 amplification (0-18.8%) Akt1 mutation (0%)

Table 1. Molecular alterations in thyroid carcinoma (from Kondo et al., 2006, modified).

has been reported in some studies, it is as yet unclear whether mtDNA mutation contributes to initiation and/or progression of thyroid cancer or only to the oncocytic phenotype. The finding of missense germ-line and somatic mutations in the GRIM19 (a nuclear gene located on chromosome 19p13.2) in oncocytic variant of FTC and PTC, but not in oncocytic adenoma or non-oncocytic carcinomas, suggests a dual function of this gene in mitochondrial metabolism and cell transformation (Maximo et al., 2005). PDTC shows loss of structural and functional differentiation, which implies they are intermediate between well-differentiated and undifferentiated thyroid carcinomas (Rosai et al., 1992; DeLellis et al., 2004; Cornett et al., 2007). Characteristically, these lesions show widely infiltrative growth, necrosis, vascular invasion and numerous mitotic figures (Figure 1E). Insular carcinomas are placed in this category. Typically, insular carcinoma is composed of small cells arranged in nests with numerous mitotic figures, necrosis, vascular invasion and infiltrative growth. ATC is composed, wholly or partially, of undifferentiated cells without the typical features of follicular-cell differentiation (Figure 1F). ATC develops from more differentiated tumors as a result of one or more dedifferentiating steps. Accordingly, half patients with ATC have either a prior or coexistent differentiated carcinoma (Rosai et al., 1992; DeLellis et al., 2004). ATC is a highly aggressive tumour, with a disease-specific mortality approaching 100% (Cornett et al., 2007). Patients with anaplastic carcinoma present with extensive local invasion, and distant metastases are found at disease presentation in 15 to 50% of patients. There is currently no effective treatment for ATC and death usually occurs within 1 year of diagnosis. ATC displays three main morphological

patterns: squamoid, pleomorphic giant cell and spindle cell. At the molecular levels, it is apparent that tumors harboring mutant BRAF and RAS are prone to progress towards PDTC or ATC. According to this hypothesis, PDTC and ATC develop from more differentiated tumors as a result of one or more dedifferentiating steps. Particularly, loss of p53 and mutations of β-catenin, which are found with increasing incidence in PDTC and ATC compared to well-differentiated tumors, may serve as a direct molecular trigger of tumor dedifferentiation (Table 1) (Nikiforova & Nikiforov 2008).

In conclusion, the simplified view of thyroid tumorigenesis depicted here holds that genetic alterations in the PI3K/AKT pathway promote thyroid cell transformation to FTC and that rearrangements in genes that encode MAPK pathway effectors seem to be required for cell transformation to PTC. Indeed, mutually exclusive, activating events that involve the genes RET/PTC, NTRK1, BRAF or RAS are detectable in nearly 70% of all PTC. By contrast, accumulation of multiple genetic alterations that can activate both pathways promotes cancer progression to ATC. This provides a strong basis for the emerging development of novel genetic-based diagnostic, prognostic, and therapeutic strategies for thyroid cancer.

2. The normal thyroid gland

The identification of the molecular properties of cancer cells is a necessary condition for the comprehension of the biology of cancer cells and, consequently, for improving diagnostic techniques and performing more efficient therapies. Tumor cells originate from normal cells that have accumulated several mutations in their DNA, and that for this reason, have acquired the capability to grow independently of the normal physiological controls and have lost, in part or totally, the ability to differentiate properly. In the normal adult thyroid gland, thyroid follicular cells represent a relatively stable cell population with a very low rate of proliferation and cell death that can be resumed in response to appropriate stimuli (Dumont et al., 1992). In humans, the adult thyroid is made of approximately 2×10^9 cells. The number of cell divisions required to generate an adult thyroid from the few precursor cells in the embryo is ~30 suggesting that each human thyrocyte divides about 5-6 times (i.e. once every 8 years) (Dumont et al., 1992). During the last decades, several cellular models that include rat thyroid cells lines as well as short-term primary cultures of dog and human thyrocytes, have been developed to investigate the mechanisms involved in the proliferation of normal thyroid cells (Medina and Santisteban, 2000; Kimura et al., 2001; Roger et al., 2010). Cell lines are simple systems that allow easy manipulation and for this reason they have represented the preferred system for *in vitro* studies of thyroid biology. Established rat thyroid cells present several properties compatible with those of "normal" differentiated thyrocytes: they are euploid, depend on TSH for growth and expression of differentiated functions, uptake iodide *in vitro*, express thyroid-specific differentiation markers (thyroglobulin, thyroperoxidase), do not grow in soft agar and are not tumorigenic in immunodeficient mice. However, several caveats must be underlined before definitive conclusions can be applied to human thyroid gland *in vivo* by extrapolating results from cultured murine or canine thyrocytes. First, the immortality itself of the cell lines indicates that they have lost some of the basic mechanisms of cell cycle control; moreover, the mechanisms that regulate cell cycle in rat, dog and human thyrocytes vary considerably (see below); finally, the effects of activated oncogenes (i.e. RAS) are sometimes very different when transfected into rat or human thyrocytes. The available data on cell cycle progression and signalling cascades involved in thyrocytes has led to the conclusion that the main regulators of thyroid growth and function are TSH and growth factors (i.e. insulin/IGF-

1). Thus it is possible to distinguish two major mitogenic pathways in thyrocytes, one that impinges on the TSHR/cAMP pathway and the other that acts through tyrosine kinase receptors of growth factors. However, the mechanisms whereby TSH/cAMP and growth factors regulate cell duplication and growth in rat, canine and human thyrocytes are mostly divergent, and will be described in detail below (Figure 2).
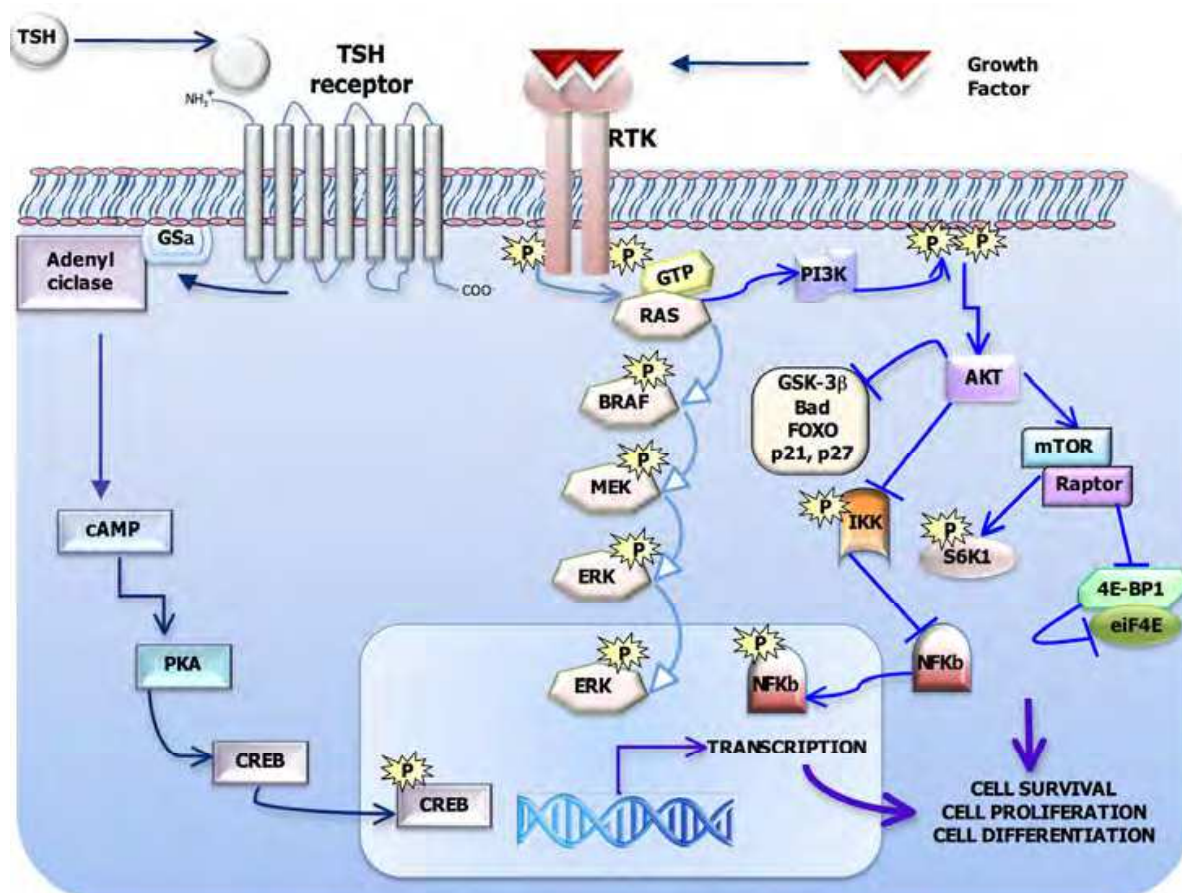

Fig. 2. Cell signalling pathways in normal thyrocytes. Thyrocytes express the TSHR and multiple growth factor receptors. TSH binds its cognate receptor and activates the G protein GSα, activating the adenylyl cyclase and increasing the level of cyclic AMP (cAMP). cAMP stimulates the cAMP-dependent protein kinase A (PKA), which in turn phosphorylates the nuclear transcription factor CREB. CREB activates the transcription of cAMP-responsive genes inducing proliferation and differentiation of thyroid follicular cells. Growth factors induce receptor-tyrosine kinase (RTK) dimerization, which results in phosphorylation of specific tyrosine residues within the cytoplasmic tail. Phosphorylated RTK activates RAS by inducing replacement of GDP with GTP. In turn, GTP-bound RAS activates the kinase BRAF and the downstream MAPK cascade. BRAF phosphorylates and activates the MAPK kinase (MEK), which phosphorylates extracellular signal-regulated kinase (ERK). Phosphorylated ERK migrates into the nucleus where it phosphorylates and activates multiple transcription factors (i.e. c-MYC, ELK1) that are involved in cell proliferation. Once activated, Akt phosphorylates a number of substrates in the cytoplasm and in the nucleus. Similarly, RTK activated PI3K signalling, which results in AKT activation. Active AKT phosphorylates and inactivates glycogen systhesis kinase-3 (GSK-3α and β), Bad, the forkhead family of transcription factors (FOXO), the CDK inhibitors p21CIP1 and p27KIP1, and conversely activate mTOR and IκB Kinases (IκKα and β).

2.1 Proliferative pathways in normal thyroid gland

So far, the most accurate model of thyroid cell cycle originated from studies performed in primary canine thyroid cells (Roger et al., 2010). Primary cultures of dog thyrocytes proliferate in monolayer culture in response to a combination of TSH, insulin, EGF and serum, though they arrest after few divisions. DNA synthesis in canine thyrocytes requires the simultaneous presence of TSH and insulin/IGF-1. Insulin or IGF-1 alone have minimal effects on DNA replication, though they support DNA synthesis and cell cycle progression induced by TSH, EGF, bFGF, or phorbol esters. By contrast, HGF is the only growth factor that acts as a full mitogen in dog thyrocytes, stimulating proliferation also in the absence of insulin/IGF-1. Established rat thyroid cell lines commonly used for the study of thyroid function and transformation are FRTL-5, PC Cl3 and WRT. FRTL-5 cells were obtained from 5-6 week old NIH Fisher 344 rats; PC Cl3 cells were obtained from 18-month old rats; WRT cells (Wistar Rat Thyroid) were established from 3-4 week old rats. Insulin/IGF-1 represents a powerful mitogen for all rat thyroid cells whereas TSH alone is not able to induce DNA synthesis in the absence of insulin it makes cells competent to respond to insulin/IGF-1, leading to the activation of MAPK and PI3K. A crucial question is how to apply the wealth of studies performed on rat and dog thyroid cells to the physiology of normal human thyrocytes. As indicated above, it appears that the canine model more accurately recapitulates the events that occur in human thyrocytes. In human primary cultures, TSH is able to induce DNA synthesis in serum-free primary cultures of adult and fetal human thyrocytes. The mitogenic effect of TSH is increased by the presence of IGF-1 or insulin, which alone weakly stimulate DNA synthesis. In thyrocytes derived from follicular adenomas, autocrine production of IGF-1 abrogates the dependence of proliferation from exogenous IGF-1. These different mitogenic stimuli exert their proliferative effects in thyrocytes by activating multiple cytoplasmic signalling cascades, which, in turn, impinge on the basic cell cycle machinery. As generally considered these mechanisms operate in the mid-to-late G1 phase of the cell cycle to promote progression through the restriction point. Typically, growth factors stimulate proliferation and inhibit differentiation. As in other cells, exposure of thyrocytes to EGF, FGF, IGF-1 or HGF activates RAS and MAPK, induces sustained expression of c-Jun and c-MYC, up-regulates cyclin D1 and down-regulates p27KIP1. On the contrary the effects exerted by TSH are in striking contrast with this general scheme. TSH induces proliferation of thyrocytes while maintaining the expression of the differentiative program. In doing so, TSH does not activate the RAS/MAPK cascade, repress c-MYC expression and increases the levels of cyclin D3 but not of cyclin D1. The differential use of cyclin D1 and cyclin D3 has been proposed to play a role in the different effects exerted by growth factors and TSH in thyrocytes (Roger et al., 2010).

2.1.1 The TSH/cAMP pathway

TSH is by far the most important physiological regulator of growth and function of thyrocytes. It is a glycoproteic hormone that recognizes a specific receptor on the thyrocyte surface, the TSH receptor (TSHR), a member of a broad class of G-protein-coupled receptors. The basic structure of these receptors comprises an extracellular segment at the N-terminus where the hormone binds, seven transmembrane helices, and three intracellular loops at the C-terminus (Vassart & Dumont, 1992). By binding to its cognate receptor TSH induces the coupling of different heterotrimeric guanine nucleotide-binding proteins (G-proteins) that include Gs, Gq/11, different subtypes of Gi and Go, G12 and G13, and cause the dissociation

of the G protein into α and βγ subunits. TSH-mediated response involves activation of Gsα, which in turn, triggering the activation of adenylate cyclase, results in increased intracellular cAMP levels. cAMP is the main second messenger in thyroid cells, and activates protein kinase A (PKA), a ser/thr kinase that is required for differentiation and proliferation of thyroid cells. Activation of PKA occurs when cAMP binds to the regulatory subunits of PKA and displaces the catalytic subunits. Once activated, PKA promotes the phosphorylation and the activation of transcription factors such as CREB (**c**AMP **R**esponse **E**lement **B**inding protein), thus inducing the transcription of genes that are required for the control of growth and differentiation of thyroid follicular cells. Proliferation and differentiation are the most important effects exerted by cAMP in thyrocytes, and are mediated by PKA activation. *In vitro*, cAMP, or agents that mimic cAMP activity such as Forskolin or 8-bromo-cAMP, stimulate expression of thyroid-specific genes, iodine uptake, synthesis and secretion of thyroid hormones, and duplication of thyroid cells. TSH or cAMP can activate also PKA-independent pathways that include the cAMP-binding GTP-exchange factors (cAMP-GEFs or Epac) that function as exchange factors for the small GTPases RAP1, RAP2, and RAS, which, in turn, activates the RAF kinases, impinging into the ERK1/2 or p38MAPK pathways. On the other hand, Gβγ subunits have been demonstrated to regulate more than 20 effectors including phospholipases, adenylyl cyclases, ion channels, G protein-coupled receptor kinases, and PI3Ks.

2.1.2 The growth factor/tyrosine kinase receptor pathway

In addition to TSH, several growth factors (i.e. EGF, HGF, FGF, IGF-1, insulin) have been shown to regulate proliferation and differentiation of thyrocytes through the establishment of autocrine and/or paracrine loops (Dumont et al., 1992; Roger et al., 2010). These factors have been shown to mediate the local action of classic hormones such as TSH (Van der Laan et al., 1995). Indeed, at least 16 receptor-type tyrosine kinases are expressed in thyrocytes, with a possible role in regulating the growth and differentiated functions of thyroid cells. Binding of tyrosine kinase receptors by the cognate ligands activates the cytoplasmic kinase domain of the receptors and triggers downstream signal transduction pathways. Activated tyrosine kinase receptors promote the recruitment of the coupling complex Shc/Grb2/SOS that catalyzes the removal of GDP from one of the RAS proteins and the loading of GTP thus promoting RAS activation. RAS are small proteins with GTPase activity, which are the upstream regulators of several signalling pathways including RAF/MEK/ERK, PI3K/AKT and RalGDS/Ral (Shields et al., 2000). The active, GTP bound RAS recruits the RAF serine/threonine kinases to cell membrane, a gene family that consists of ARAF, BRAF and RAF-1 (CRAF). In turn, active RAF proteins phosphorylate and activate the Mitogen-activated protein kinase/Extracellular signal-regulated Kinases (MEKs), which phosphorylate and activate the serine/threonine **E**xtracellular-signal-**r**egulated **k**inases 1,2 (ERK). ERKs directly phosphorylate many transcription factors including Ets-1, c-Jun and c-Myc. ERKs can also phosphorylate and activate the 90 kDa ribosomal S6 kinase (p90[Rsk]), which then leads to the activation of the transcription factor CREB (Shields et al., 2000). By altering the levels and activities of transcription factors, the MAPK pathway leads to altered transcription of genes that are important for the cell cycle. Many growth factors receptors such as PDGFR, EGFR, IGF-1R and insulin receptor activates also the PI3K/AKT pathway (Engelman et al., 2006). Accordingly, in thyroid cells IGF-1, EGF and HGF induces phosphorylation and activation of AKT and p70S6 (p70S6K) kinases downstream of

phosphatidylinositol-3-kinase (PI3K). After ligand-induced activation of specific receptors, PI3K can be activated through one of two different mechanisms. First, activation of tyrosine kinase receptors generates phosphorylated tyrosine residues on the receptor that serve as docking sites for the p85 regulatory subunit of PI3K, which then recruits the p110 catalytic subunit to the complex, thus triggering downstream signalling. PDGFR and insulin receptor that have binding sites for p85 strongly activate PI3K upon binding to their ligands. Alternatively, GTP-bound RAS can activate PI3K by direct interaction with the catalytic subunit (Brasil et al., 2004). Activated PI3K converts phosphatidylinositol 4,5 biphosphate (PtdIns-3,4-P3) into phosphatidylinositol 3,4,5 phosphate (PtdIns-3,4,5-P3), which resulting in membrane localization of phosphoinositol-dependent kinase-1 (PDK1) via its pleckstrin homology (PH) domain. AKT is also recruited to the 3' phosphorylated phosphatidylinositol-rich plasma membrane by its PH domain, where it is fully activated by phosphorylation at residues T308 and S473 by PDK1 and TORC2 complex, respectively. AKT is the primary mediator of PI3K-initiated signalling. Conversely, the PTEN and SHIP-1/2 phosphatases that remove the phosphate group from the 3' position of the inositol ring of PtdIns-3,4,5-P3 are responsible for turning off PI3K signalling and antagonizing the activity of AKT (Carracedo & Pandolfi, 2008). AKT activation plays a fundamental role in the regulation of glucose metabolism, cell migration proliferation and survival by phosphorylation of a number of downstream substrates. Among these targets are: Bad, Bim, procaspase-9, IκKalpha, the forkhead family of transcription factors FOXO1, FOXO3a, GSK-3β, the ubiquitin ligases MDM2 and SKP2, the CDK inhibitors p21CIP1 and p27KIP1 and others. It is worth noting that AKT can either cause the activation of specific substrates (*e.g.*, MDM2, IκKalpha and CREB) or may mediate the inactivation of other proteins (*e.g.*, RAF, BRAF, p21CIP1, p27KIP1, BIM, BAD, procaspase-9, FOXO3a, and GSK-3β) (Manning & Cantley, 2007).

2.2 Biochemical aspects of signal transduction and cell cycle regulation in normal thyrocytes

Growth factors and TSH regulate cell cycle progression of thyrocytes with apparently different mechanisms (see Roger et al., 2010). In dog thyrocytes TSH does not activate RAS, PI3K, AKT or the different MAPKs but it activates mTOR. Conversely, insulin/IGF-1 strongly activates RAS, PI3K, AKT and the MAPKs. Interestingly, the observation that HGF, the ligand of the tyrosine kinase receptor MET, is the only growth factor that is able to stimulate both the MAPK- and the PI3K-dependent pathways, possibly explains why HGF is the only growth factor that acts as a full mitogen in dog thyrocytes, stimulating proliferation also in the absence of insulin. In dog thyroid cells, pRB phosphorylation is the critical event that regulates the passage through the restriction point. It has been convincingly shown that the complementary action of TSH and insulin converge on the activation of cyclin D3-CDK4 complexes, whose activity is required for pRB phophorylation and DNA synthesis in response to TSH and insulin. However, TSH-mediated proliferation of dog thyrocytes requires cyclin D3 and is independent of down-regulation of the cyclin-dependent kinase (CDK) inhibitor p27KIP1 whereas cyclin D3 is not required for growth factor-dependent proliferation. The current model holds that TSH (and cAMP) permits the passage through the restriction point by acting on the assembly, nuclear translocation and phosphorylation of an active cyclin D3-bound CDK4. This results in the redistribution of p27KIP1 from cyclin E/CDK2 to cyclin D3/CDK4 complexes, presumably allowing CDK2

activation and pRB phosphorylation (Roger et al., 2010). Conversely, IGF-1 or HGF induce cell cycle progression along G1 by increasing the levels of cyclin D1 and reducing those of p27KIP1.

Rat FRTL-5 cells proliferate rapidly (doubling time ~36-40 h) in the presence of serum and a six-hormone mixture (6H) containing TSH and high concentrations of insulin (that activate also IGF-1 receptors) (Medina & Santisteban, 2000). Insulin/IGF-1 are the only genuine mitogens for FRTL-5 whereas TSH makes cells competent to respond to insulin/IGF-1. bFGF, HGF as well as EGF are all able to induce robust DNA synthesis in synergy with TSH or insulin. In FRTL-5 cells proliferation induced by TSH or by cAMP requires RAS, AKT and PI3K signalling (Cass & Meinkoth, 2000; Ciullo et al., 2001). RAS activity is apparently necessary for TSH to induce the transition from quiescence to G1, though the ERK pathway seems not involved. Conversely, cAMP activates PKA and at the same time, influences the selection of RAS effectors (PI3K versus RAF). According to this model, PKA-phosphorylated p85 stabilizes the complex p110-p85 and thus facilitates the interaction between PI3K and RAS. In parallel, cAMP inhibits RAF/ERK signaling by decreasing RAF availability to RAS. Under these circumstances cAMP increases PI3K signaling (De Gregorio et al., 2007; Cosentino et al., 2007). Other studies have demonstrated that TSH/cAMP is able to activate ERKs and p38 MAPK, as well as to induce cyclin D1 and down-regulate the cyclin-dependent kinase inhibitor p27KIP1. Other rat thyroid cell lines – namely WRT and PC Cl3 cells - present discrepancies with FRTL-5. Similar to FRTL-5 cells, PC Cl3 cells are routinely maintained in a medium containing TSH and insulin. Insulin/IGF-1 stimulate proliferation and growth in size of PC Cl3 cells, and this effect is amplified by TSH (Kimura et al., 2001). Activation of the PI3K pathway by TSH in rat thyrocytes (WRT) and the involvement of cAMP in this pathway are controversial and depend on the specific cell type. In fact, TSH treatment leads to release of Gβγ dimers and subsequent activation of PI3K, one of the putative effectors of Gβγ dimers. Although debated, TSH has been shown to activate RAS and PI3K in WRT cells (Tsygankova et al., 2000). On the other hand, interference with RAS or PI3K activity impairs TSH-stimulated DNA synthesis. Through the activation of these pathways, TSH and serum deplete nuclear stores of p27KIP1, allowing activation of nuclear CDK2 and entry into S phase. TSH and serum regulate p27KIP1 in very different ways: TSH stimulated the nuclear accumulation of p27KIP1, whereas serum induced its nuclear export (Medina & Santisteban, 2000). DNA synthesis of PC Cl3 cells is also induced by FGF, phorbol esters (either in the presence or not of insulin) but not by EGF or HGF. WRT cells apparently proliferate in response to the activation of either the TSH/cAMP or insulin/IGF-1 cascades but are unresponsive to TPA, EGF and HGF (Roger et al., 2010). The PI3K pathway mediates most of the effects exerted by insulin/IGF-1 on cell cycle progression in rat thyrocytes. In fact, PI3K inhibitors impair insulin/IGF-1-dependent DNA synthesis and block the ability of insulin/IGF-1 to reduce p27KIP1 expression, to induce expression of cyclins D1 and E and to phosphorylate pRB (Roger, 2010). In serum-free primary cultures of adult and fetal human thyrocytes, TSH is able to induce DNA synthesis. However, the stimulation of DNA synthesis and/or proliferation by TSH decreases if thyrocytes originate from old people or cells exposed to high serum concentrations. In monolayer cultures, the effect of TSH is mimicked in large part, though not totally, by cAMP enhancers (forskolin, cholera toxin, (Bu)2 cAMP), with the mitogenic effect of TSH being increased by the presence of IGF-1 or insulin, which alone weakly stimulate DNA synthesis. In the absence of

exogenous insulin or IGF-1, the TSH-dependent DNA synthesis in human thyrocytes cultured with 1% serum is weak and depends on autocrine IGF production. The autocrine production of IGF-1 is further increased in thyrocytes derived from follicular adenomas, which abrogate dependence of proliferation from exogenous IGF (Roger, 2010).

3. Molecular biology of thyroid cancer

Cancer is a genetic disease in the sense that it affects genes. In the past decades many genes that have a causal role in thyroid cancer have been discovered and the pathways through which they act have been elucidated in their basic structures (Kondo et al., 2006; Nikiforova & Nikiforov 2008; Xing, 2008). The identification of the biochemical functions of these genes has allowed to highlight a small number of subverted pathways in follicular cell-derived tumors. Using both cell culture systems and experimental murine models of cancer it has become apparent that the malignant transformation of the thyroid follicular cell involves multiple genetic events that sequentially activate certain oncogenes (i.e. RAS, RET/PTC, NTRK1, BRAF, PIK3CA, AKT1) and inactivate specific tumour suppressors (i.e. p53, PTEN). These recurrent alterations are frequently mutually exclusive and occur in genes within relatively few critical pathways such as the TSH/cAMP, MAP kinase and the PI3K/AKT signalling cascades (Figure 3). The mitogenic and differentiating TSH/cAMP pathway is involved in hyperthyroidism whereas the mitogenic dedifferentiating growth factor-regulated MAPK pathway is involved in the development of thyroid cancer. On the other hand, recent evidences indicate that the constitutive activation of the PI3K/AKT pathway is implicated in the development of differentiated and poorly differentiated carcinomas.

3.1.1 The TSH/cAMP Pathway: Hyperfunctioning adenomas

As indicated, the TSH/cAMP pathway is the major regulator of follicular cell proliferation and function. Expectedly, the constitutive activation of this pathway plays a critical role in the pathogenesis of benign hyperfunctioning thyroid nodules and adenoma. Adenoma frequently displays gain-of-function mutations that confer constitutive activity to TSHR in 50–80% or GSα in 8% of cases, respectively. TSHR is encoded by a gene located on chromosome 14q31; GSα is encoded by GNAS1 gene located on chromosome 20q13. Similarly, mutations in TSHR or GNAS1 genes account for hyperfunctioning nodules in patients with multinodular goiters (Khron et al., 2005; Parma et al, 1993). Dominant activating mutations of the TSHR are also the cause of non-autoimmune hyperthyroidism, a common thyroid disorder. In adenoma, mutations are somatic and strongly activate the cAMP cascade in one cell, thus initiating a clonal expansion of the mutated cell that lead to autonomous tumor growth. Germline GNAS1 mutations are responsible for the McCune–Albright syndrome, a familial condition that include hyperthyroidism and growth hormone excess. In addition, inactivating mutations in the gene encoding PKA type 1-alpha regulatory subunit (PRKAR1A), have been identified in the Carney Complex syndrome, an autosomal dominant disease comprising myxomas of the heart and skin, hyperpigmentation of the skin and endocrine overactivity that has features overlapping those of the McCune–Albright syndrome (Lindor & Greene, 2008). The mutations of TSHR and GSα constitutively activate adenylyl cyclase leading to increased cAMP accumulation and TSH-independent proliferation. However, adoptive expression of TSHR induces neoplastic transformation of FRTL-5 cells as demonstrated by growth in semi-solid medium and tumorigenesis in nude

mice whereas GSα does not. Accordingly, the constitutive activation of the cAMP cascade alone is apparently insufficient for the malignant transformation of thyroid follicular cells because: i) mutations of TSHR or GNAS1 are rarely detected in well-differentiated carcinomas; ii) hyper-functioning thyroid nodules rarely become malignant; and iii) patients with the McCune–Albright syndrome, which result from germline GNAS1 mutations, present low-incidence of thyroid cancer (Collins et al., 2003).
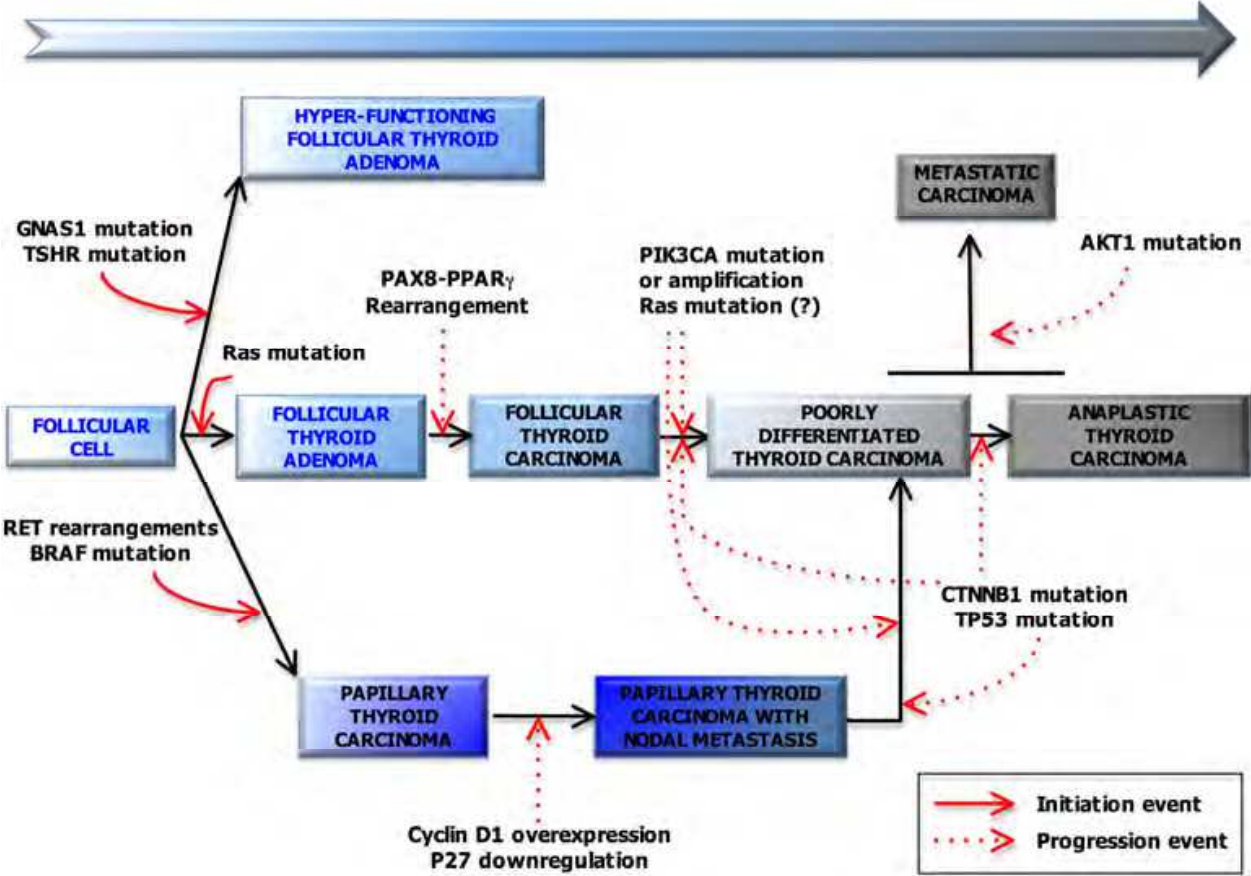

Fig. 3. The stepwise mechanism of thyroid carcinogenesis. Three distinct pathways have been proposed for the initiation of thyroid tumors including hyper-functioning follicular thyroid adenoma, FTC and PTC. Genetic defects that result in activation of RET or BRAF represent frequent early initiating events associated with radiation exposure that lead to PTC development. RAS mutations represent frequent early initiating events, associated with iodine deficiency, that lead to FTC development. By contrast, most PDTC and ATC are considered to derive from pre-existing well-differentiated thyroid carcinoma through the accumulation of additional genetic events that include nuclear accumulation of β-catenin (encoded by CTNNB1) and p53 inactivation.

Finally, more solid evidence on the role of the TSH/cAMP pathway in the transformation of thyroid follicular cells come from the study of transgenic mice (Kim and Zhu, 2009 and references therein). Murine strains modelling the constitutive activation of the cAMP-dependent mitogenic cascade in the thyroid gland provokes a phenotype very similar to the one seen in humans, with development of hyperplasia but not of overt tumors. In addition,

mice made hypothyroid with antithyroid drugs do not develop thyroid cancer despite dramatic increase in serum TSH levels. Similarly, transgenic mice expressing the canine adenosine A2 receptor, which signals through G proteins and activates PKA as cAMP does, develop goiters and hyperthyroidism, but not thyroid cancer. Other mouse models that mimic TSHR overactivation via constitutive activation of GSα under control of the Tg promoter or with thyroid-specific expression of cholera toxin A1 subunit, develop goiters and hyperthyroidism, but not thyroid cancer. Finally, in a mouse model of PKA overactivation mice that are heterozygous for a null allele of the type 1a regulatory subunit of PKA (Prkar1a), develop PTC only sporadically and with long latency.

3.1.2 The RTK/RAS/BRAF/MAP kinase pathway: Papillary thyroid carcinomas

The most studied pathway involved in thyroid tumorigenesis is the RTK/RAS/BRAF/MAP kinase pathway, which seems to be essential for the development of PTC but apparently plays a more limited role in FTC. As in other tumors, these genetic events are mutually exclusive, providing compelling evidence for the requirement of this signalling system in PTC development (Figure 3).

Tyrosine kinase receptors

Tyrosine kinase receptors of growth factors regulate critical cellular functions required for tissue homeostasis such as cell proliferation, differentiation, survival, and apoptosis. Not surprisingly, signalling through these receptors is considered essential for initiation and progression of a broad spectrum of human tumours. Accordingly, certain subtypes of thyroid carcinomas are characterized by the aberrant activity of receptor-type tyrosine kinases (RET, NTRK1) that is consequent either to chromosomal rearrangements or to overexpression (EGFR, MET) (Kondo et al., 2006; Nikiforova & Nikiforov 2008; Xing et al., 2008). RET was the first activated receptor-tyrosine kinase to be identified in thyroid cancer. The RET proto-oncogene is located on chromosome 10q12 and encodes a tyrosine-kinase receptor protein with four cadherin-related motifs in the extracellular domain and a kinase in the cytoplasmic domain, whose expression and function is normally restricted to a subset of cells derived from the neural crest. RET is not normally expressed in follicular cells but is expressed in the developing central and peripheral nervous systems and is required for renal organogenesis and enteric neurogenesis (Fusco & Santoro, 2007). RET ligands include the glial cell line-derived neurotrophic factor (GDNF), and GDNF-like proteins such as Neurturin, Persephin, and Artemin. GDNF and GDNF-like proteins signal through a multi-component receptor system including the GPI-linked membrane receptor GDNF Family Receptors alpha (αGFRs), whose function is to bind the ligands and present them to the receptor, and RET, which operates as an intracellular signal transducing element (Airaksinen et al., 1999). RET activation is followed by dimerization, autophosphorylation at selected tyrosine residues and engagement of effectors through specific phosphorylated tyrosines. Activated RET triggers several downstream signal-transduction pathways including MAPK, PI3K and JNK (Fusco & Santoro, 2007). Different sites of tyrosine phosphorylation in the RET protein have been identified as docking sites for signalling molecules: Y905 that map in the kinase A loop mediates the recruitment of the SH2 domain-containing proteins Grb7 and Grb10; Y1015 mediates the association with phospholipase Cγ (PLCγ); and Y1062 that interacts with Shc and Frs2, which in turn, mediate RAS/RAF/MAPK activation. However, neither Y1015 nor Y1062 alone are apparently required for RET/PTC-induced effects on growth and apoptosis whereas, by contrast, there is an absolute requirement of Y1062 for RET/PTC-induced dedifferentiation (Knauf et al., 2003).

Chimeric oncogenes designated RET/PTC have been implicated in the development of PTC (Figure 4)(Fusco & Santoro, 2007). The RET/PTC oncogene is generated by chromosomal rearrangements resulting in the fusion of the RET tyrosine-kinase domain to the 5'-terminal region of heterologous genes. All rearrangements appear to be balanced inversions or translocations that involve the 3.0 kb intron 11 of RET. The RET/PTC rearrangement results from a fusion between the 3'-portion of RET that leaves intact the tyrosine kinase domain and the 5'-portion of various heterologous genes. All RET-fused genes provide putative dimerization domains to the chimeric RET/PTC genes. RET/PTC chimeric oncoproteins lack the signal peptide and the transmembrane domain, are expressed in the cytoplasm of follicular cells under the control of the newly acquired promoters, and show constitutive dimerization and ligand-independent activation of RET tyrosine kinase, which is essential for the transformation of thyroid cells. To date, at least 15 chimeric genes have been reported (Fusco & Santoro, 2007). The most common rearrangements are RET/PTC1, RET/PTC3 and RET/PTC2, respectively. RET/PTC1 and RET/PTC3 are generated by paracentric inversions at 10q between RET and H4 (OMIM #601985) or NCOA4 (ELE1) (OMIM 601984), respectively. RET/PTC2 is due to an interchromosomal translocation between chromosome 10 and chromosome 17 (Fusco & Santoro, 2007). Among human tumours, RET/PTC rearrangements were initially associated with PTC, radiation exposure and young age (Santoro et al., 1992; Ito et al., 1994). Reported frequencies of RET/PTC rearrangements in sporadic PTC vary widely among different countries. Depending on the detection method used and/or the geographical location of patients the frequency of RET/PTC rearrangements varies from 3% in Saudi Arabia to 59% in the United Kingdom; however a reasonable estimates of the frequency of RET/PTC rearrangements in adult patients is ~20%, with higher values in patients with a history of radiation exposure (50–80%). The high prevalence of RET/PTC rearrangements in children from the areas affected by nuclear disaster at Chernobyl indicates a role for radiation damage in the genesis of these paracentric inversions (Fusco & Santoro, 2007). Accordingly, exposure of cell lines to ionising radiation results in the expression of RET/PTC within hours, supporting a direct role for radiation in the recombination of RET (Ito et al., 1993).

There is compelling evidence that different RET/PTC rearrangements present variable oncogenic potential. Different types of RET/PTC are associated with distinct subtypes of PTC. RET/PTC1 tends to be more common in small indolent tumours with typical papillary growth and to have a more benign clinical course, whereas RET/PTC3 shows a strong correlation with subtypes believed to represent aggressive forms of papillary cancer such as the solid variant and, more recently, the tall cell variant (Nikiforov et al., 1997; Basolo et al., 2002). Accordingly, transgenic mice expressing RET/PTC1 under the control of the rat Tg promoter developed PTC (<50%) with a long latency period and with no distant metastasis. Similarly, transgenic mice expressing RET/PTC1 under the control of the bovine Tg promoter developed PTC. However, in both mouse strains metastases were absent indicating that RET-PTC1-depended cancers requires additional mutations (i.e. knockout of the tumor-suppressor p53) to result in metastasis (Kim and Zhu, 2009 and references therein). By contrast, RET/PTC3 mice develop PTC-like lesions that are similar to the human solid variant of PTC, and unlike RET-PTC1 mice, in about one-third of cases, develop axillary lymph node metastasis (Kim and Zhu, 2009 and references therein).

Although transgenic mouse models have shown that RET/PTC rearrangements can initiate thyroid carcinogenesis *in vitro*, the same studies have indicated that RET/PTC

represents a weak tumour-initiating event, requiring additional genetic and/or epigenetic changes for clonal expansion of mutated cells. RET/PTC expression in thyroid cells induces dedifferentiation and apoptosis at the same time. However, at difference with RAS and BRAF, RET/PTC rearrangements do not induce genomic instability. Moreover, TSH-independence may develop in RET-positive tumours as a secondary adaptation during cancer progression since it has been shown that RET/PTC-transfected cells can acquire the capability to grow in a TSH-independent manner. Additional evidence demonstrating that RET/PTC rearrangements are tumour-initiating events is that they are present in microcarcinomas. Indeed a high frequency of RET/PTC rearrangements have been reported in 42-77% of the subclinical microcarcinomas detected at autopsy or in thyroidectomies for disorders other than cancer. In addition, RET alterations have been found in other early benign lesions such as follicular adenomas, benign thyroid nodules and Hashimoto's thyroiditis. The high frequency of RET rearrangements in microcarcinomas and in early benign lesions is consistent with the idea that they represent early events in the neoplastic processes. On the other hand, the low prevalence of RET rearrangements in poorly differentiated and undifferentiated thyroid carcinoma supports a minor role for RET/PTC in tumour progression (Fusco & Santoro, 2007).

The neurotrophic receptor-tyrosine kinase NTRK1 (also known as TRK and TRKA) was the second identified gene subjected to chromosomal rearrangement in thyroid cancer (Pierotti et al., 2001). The NTRK1 proto-oncogene is located on chromosome 1q22 and encodes the transmembrane tyrosine-kinase receptor for nerve growth factor (NGF). NTRK1 expression is typically restricted to neurons and regulates neuronal growth and survival. The activated receptor initiates several signal-transduction cascades including ERK, PI3K and the phospholipase-Cγ (PLCγ) pathways (Miller & Kaplan, 2001). Similar to RET, NTRK1 is activated in thyrocytes by chromosomal rearrangements that fuse the NTRK1 tyrosine kinase domain to the 5'-terminal region of heterologous genes. NTRK1 rearrangements have been detected in 5–13% of sporadic PTC but only in 3% of post-Chernobyl childhood PTC (Bongarzone et al., 1996). To date, three different rearrangements have been identified as chimeric oncogenes. The recombination events that cause the oncogenic activation of NTRK1 include an inversion fusing NTRK1 to non-muscular tropomyosine (TPM3) gene located at 1q31, a different intra-chromosomal rearrangement that juxtaposes NTRK1 to the 5'-end of a translocated promoter region (TPR) gene localized at 1q25 or to the 5'-sequence of a TRK-fused gene (TFG) localized on chromosome 3 (TRK-T1, TRK-T2 and TRK-T3 oncogenes, respectively). In all cases the resulting chimeric proteins exhibit ectopic expression and constitutive activation of the tyrosine kinase (Pierotti et al., 2001). The prevalence of each fusion type is nearly equal in sporadic PTC, whereas TPM3–NTRK1 is more frequent than other NTRK1 rearrangements in post-Chernobyl childhood PTC. The generation of TRK-T1 transgenic mouse model have demonstrated that, in contrast with *in vitro* results, TRK-T1 can initiate thyroid cancer. About half of the transgenic mice that expressed TRK-T1 developed thyroid cancer, either FTC or PTC, without distant metastasis (Kim and Zhu, 2009).

The receptor-tyrosine kinase MET (which is located on chromosome 7q31) encodes a two-subunit 190 kDa transmembrane protein that is the receptor for HGF. HGF is a powerful mitogen for thyrocytes and modulates thyroid cancer cell motility and invasiveness and promotes angiogenesis. MET is often overexpressed in PTC (77–93%),

but is rare in other histological types of thyroid tumours (Di Renzo et al., 1995), though the pathogenetic significance of MET expression in papillary thyroid cancer remains to be identified. Some studies found MET overexpression associated with advanced tumor stages of thyroid carcinoma and histologic variants associated with poor prognosis while others showed decreased MET expression in poorly or undifferentiated tumors with an inverse correlation between MET expression and vascular invasion and distant metastases (Di Renzo et al., 1995). On the other hand, the finding that stromal cells of the thyroid secrete HGF suggests that MET may be involved in the stimulation of tumor growth through a paracrine mechanism. MET overexpression is apparently due to transcriptional or post-transcriptional mechanism. For example oncogenic RAS and RET/PTC have been shown to induce MET overexpression in thyroid follicular cells. Point mutations involving MET have also been detected in about 7% of well-differentiated thyroid carcinoma.

The epidermal growth factor receptor (EGFR) family includes EGFR (also known as ERBB1 or HER1), ERBB2 (also known as HER2), ERBB3 (also known as HER3) and ERBB4 (also known as HER4). All are involved in the transmission of signals that control cell growth and differentiation. Multiple ligands bind EGFR, ERBB3 or ERBB4, inducing rapid receptor dimerization, with a marked preference for ERBB2 as dimerization partner. EGFR and ERBB2 are often found in thyroid cancers (Kato et al., 2004). EGF stimulates the growth of human thyroid carcinoma cells and rat FRTL-5 cells *in vitro*. At difference with lung and breast carcinomas where EGFR mutations or ERBB2 amplification have been reported, respectively, neither activating mutations nor DNA amplification of EGFR were found in thyroid cancer. Conversely, thyroid tumors overexpress EGFRs and ligands, implicating EGFR signalling in thyroid tumorigenesis. Increased expression of EGFR correlates with poor prognosis in differentiated thyroid cancers whereas ERBB2 has no clear prognostic significance.

The Fibroblast growth factors (FGFs) and FGF receptors (FGFRs) are important regulators of angiogenesis and tumorigenesis (Grose & Dickson, 2005). At least 20 FGF ligands that signal through a complex family of receptor-tyrosine kinases, encoded by four distinct FGFR genes exist. So far, no mutations or rearrangements that involve members of the FGFR family have been identified in thyroid cancer. Conversely, FGFR1, FGFR3 and FGFR4 are overexpressed in thyroid carcinoma with FGFR4 expression restricted to the aggressive forms of thyroid carcinoma (St Bernard et al., 2005). In addition, the adoptive expression of FGFR3 in a human thyroid carcinoma cell line results in aberrant growth. As to the growth factors, expression of FGF2 (also known as basic FGF) is apparently increased in thyroid cancer and promotes mitogenic activity of rat thyroid follicular cells.

Vascular endothelial growth factor (VEGF) ligands — VEGFA, PlGF, VEGFB, VEGFC and VEGFD — are angiogenic growth factors that, by binding their cognate receptors on vascular cells, induce proliferation of endothelial and/or lymphatic cells. VEGFA, PlGF and VEGFB stimulates angiogenesis, whereas VEGFC and VEGFD promotes lymphangiogenesis (Bunone et al., 1999). Increased expression of VEGFA and PlGF has been frequently reported in thyroid goiters and carcinomas (Bunone et al., 1999). Conversely, the overexpression of VEGFC and VEGFD are implicated in development of the lymphatic system and correlates with the density of lymphatics and lymph-node metastasis of PTC (Hung et al., 2003).
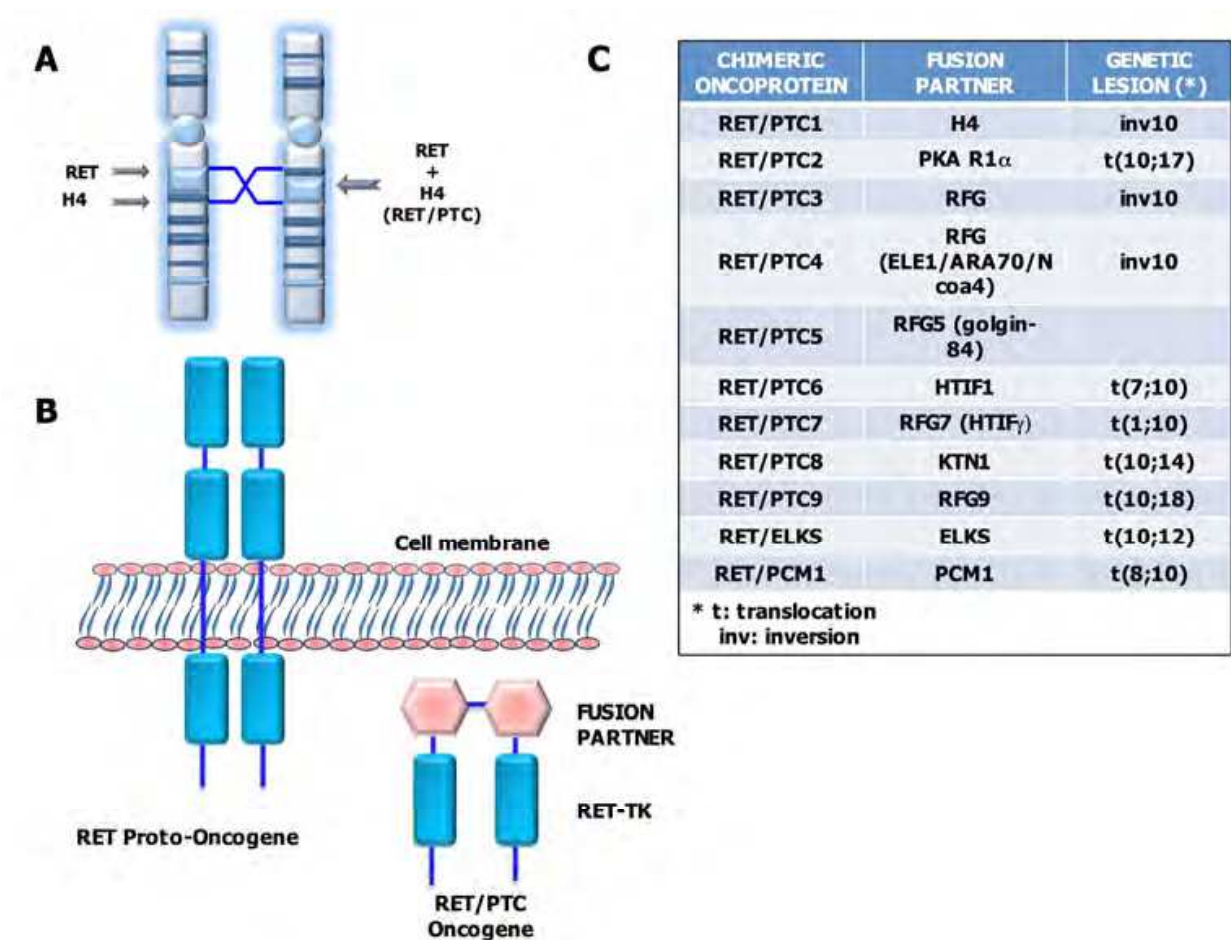
CHIMERIC ONCOPROTEIN	FUSION PARTNER	GENETIC LESION (*)
RET/PTC1	H4	inv10
RET/PTC2	PKA R1α	t(10;17)
RET/PTC3	RFG	inv10
RET/PTC4	RFG (ELE1/ARA70/Ncoa4)	inv10
RET/PTC5	RFG5 (golgin-84)	
RET/PTC6	HTIF1	t(7;10)
RET/PTC7	RFG7 (HTIFγ)	t(1;10)
RET/PTC8	KTN1	t(10;14)
RET/PTC9	RFG9	t(10;18)
RET/ELKS	ELKS	t(10;12)
RET/PCM1	PCM1	t(8;10)

* t: translocation
inv: inversion

Fig. 4. RET/PTC rearrangements in papillary thyroid carcinoma. A. Schematic representation of the molecular mechanism that generates PTC oncogene. B. A comparison between the RET proto-oncogene and the RET/PTC oncogene. C. A list of the different RET/PTC rearrangements identified.

The RAS G-protein

The RAS protooncogenes encode 21 kDa monomeric G-proteins, which transduce signals from a wide variety of growth factor receptors, particularly those of the tyrosine kinase family. Three RAS proto-oncogenes — HRAS (which is located on chromosome 11p11), KRAS (which is located on chromosome 12p12), and NRAS (which is located on chromosome 1p13) — are implicated in human cancer (Buday & Downward, 2008). The three RAS genes encode highly related proteins with GTPase activity that are located at the inner surface of the cell membrane and play a central role in the intracellular transduction of signals arising from cell membrane. In its inactive state, RAS is bound to guanosine diphosphate (GDP). Upon activation, it releases GDP, binds guanosine triphosphate (GTP), thus transiently activating downstream signalling and terminates signalling by hydrolizing GTP. RAS proteins convey signals from tyrosine kinase receptors and G-protein-coupled receptors (GPCRs) to different signalling pathways such as MAPK, PI3K and Ral-GDS, which activate the transcription of target genes resulting in the regulation of cell proliferation, migration and survival (Peyssonnaux & Eychene, 2001). Point mutations occurring in tumors affect the guanosine triphosphate (GTP)-binding domain (codons 12/13) or the GTPase domain (codon 61) and result in the replacement of specific amino

acid residues that lock p21RAS in a constitutively active form of the protein. Such gain-of-function RAS mutations promote tumor development. Accordingly, it is estimated that around 30% of all human tumours contain a mutation in a RAS allele, which makes RAS genes the most mutated proto-oncogene in the human genome.

Oncogenic mutations involving all three RAS genes were among the first genetic alterations to be identified in tumours originating from the thyroid follicular epithelium and have been reported with variable frequency in thyroid neoplasms ranging from 7 to 62% (Vasko et al. 2003). Initially it has been proposed that RAS mutations might represent one of the early steps in the formation of thyroid cancer because they have been observed in benign tumours. However, more recent studies have demonstrated that RAS mutations are more represented in PDTC (55%) and ATC (52%) than in follicular adenomas and WDTC (5 to 10%), and that there exists a significant association between RAS mutations and poor survival (Garcia-Rostan et al., 2003). Although RAS mutations are not restricted to a specific thyroid tumour type they are more common in iodine-deficient and in lesions with follicular architecture, including FTC and follicular variant of PTC, and are rare in radiation-induced thyroid cancers of Chernobyl. RAS mutations are thought to be among the initiating molecular events in thyroid tumorigenesis. RAS mutants are able to activate both the PI3K/AKT and MAPK signalling cascades and, conversely, oncogenic transformation by mutant KRAS requires activation of both MAPK and PI3K/AKT pathways. Adoptive expression of HRAS-V12 into cultured rat thyroid cells promotes TSH-independent growth and dedifferentiation as a result of inhibition of the activity of TTF1 and PAX8, two transcription factors essential for the maintenance of the thyroid differentiated state (De Vita et al., 2005). By contrast, adoptive expression of mutant RAS into human thyrocytes stimulate growth and differentiation (Gire et al., 2000). RAS activation in rat PC Cl3 cells displays also evidence of DNA damage, manifesting as chromosome misalignment, centrosome amplification and micronuclei formation and increased susceptibility to apoptosis (Saavedra et al., 2000). In the presence of TSH, HRAS-G12V also triggers the initiation of programmed cell death but, in the absence of TSH, acute expression of mutant RAS inhibits apoptosis and accelerates TSH-independent proliferation. The cells that loose TSH responsiveness and, at the same time, inactivate the RAS-dependent apoptotic cascade will undergo clonal expansion and tumor development (Shirokawa et al., 2000).

In vivo studies with transgenic mice have shown controversial results on the role of RAS in thyroid carcinogenesis (Kim and Zhu, 2009 and references therein). In some reports, mutant HRAS or KRAS alone are not apparently sufficient to induce cancer and it appears that additional genetic alterations are required for FTC development. Similarly, mice carrying mutant KRAS-G12V under the control of rat Tg promoter or KRAS-G12D under control of the endogenous KRAS promoter showed no sign of thyroid cancer, though another transgenic mouse strain expressing a mutated HRAS-G12V controlled by the bovine Tg promoter developed PTC. Conversely, targeting human NRAS with a mutation at codon 61 to thyroid follicular cells induced, in 30% of the transgenic mice, progressive changes from hyperplasia to adenoma and carcinoma that were of follicular or mixed histotype with large poorly differentiated areas closely resembling those observed in human patients.

The serine/threonine kinase BRAF

The proto-oncogene BRAF situated on 7q24 encodes a serine/threonine kinase that transduces regulatory signals through the RAS/RAF/MEK/ERK cascade. There are three isoforms of the RAF kinases in mammalian cells: ARAF, BRAF, and CRAF (also denoted

RAF1). BRAF is more efficient in phosphorylating MEKs than other RAF isoforms. RAF proteins play a critical role in the transduction of signals by growth factors, hormones and cytokines, being involved in the regulation of cell proliferation, differentiation and apoptosis (Peyssonnaux & Eychene, 2001). Expectedly, gain-of-function BRAF mutations provide an alternative route for the aberrant activation of ERK signalling that is implicated in the tumorigenesis of several human cancers — for example, melanoma and colon carcinoma (Davies et al., 2002). BRAF mutations represent the most common genetic change in PTC, having been detected in 29–83% PTC, especially in the aggressive tall-cell variant (55–100%), but not in FTC. In addition, BRAF mutations have also been observed in up to 13-15% of PDTC and 35% of ATC. By contrast, BRAF mutations are a relatively rare event in post-Chernobyl and sporadic childhood PTC. Interestingly, the frequency of BRAF mutations in ATC arising from pre-existing PTC is significantly higher than those arising from pre-existing FTC (Nikiforova et al., 2003). BRAF mutations are almost always exclusive to RAS genes mutations as well as to RET (RET/PTC) and NTRK1 rearrangements, altogether accounting for about 70% of PTC cases. BRAF mutations in PTC correlate with more advanced clinical stage, extrathyroidal extension and distant metastasis (Xing et al., 2005). Moreover, tumors with BRAF mutations are apparently unresponsive to ^{131}I treatment, pointing out that this genetic event is a new biological marker that predicts poor prognosis and resistance to treatment (Xing et al., 2005). This is consistent with the notion that BRAF mutations in human PTC are associated with decreased expression of iodine-metabolising genes (i.e. NIS, pendrin, Tg) and that, in addition, the conditional expression of BRAF-V600E in rat thyrocytes promotes down-regulation of TSHR, NIS, Tg, TTF-1 and PAX-8.

The great majority of BRAF mutations detected in PTC (>90%) are of a single type: a 1799T-A transition in exon 15 leading to the substitution of a valine by a glutamic acid at the position 600 (V600E), which one of the most prevalent somatic genetic events in human cancer (Figure 5). The V600E mutation of BRAF destabilise the inactive BRAF structure by generating repulsive electrostatic forces in the activation loop, thereby leading to a constitutive catalytic activation that stimulates ERK activity and transforms NIH3T3 cells. Interestingly, whereas the V600E mutation is common in classical and tall cell variant of PTC, the K601E mutation has been detected in the follicular variant. An alternative alteration of BRAF detected in radiation-associated thyroid cancers is a chromosomal rearrangement of BRAF (AKAP9–BRAF) (Ciampi et al., 2005). AKAP9-BRAF results from a paracentric inversion of the long arm of the chromosome 7 and leads to the fusion of the first 8 exons of the A-kinase anchor protein 9 (AKAP9) gene with the C-terminal coding region of the BRAF protooncogene. This fusion leads to a chimeric protein with constitutively activated BRAF kinase. The AKAP9-BRAF rearrangement has been reported in about 11% of post-Chernobyl, radiation-associated PTC whereas only 1% of sporadic PTC displays this mutation. Regardless of the mode of activation, these data highlight the crucial contribution of BRAF as an important effector in the role of MAPK activation and in thyroid tumorigenesis. BRAF mutations are thought to be a tumour-initiating event. BRAF concomitantly induces stimulation of DNA synthesis and apoptosis, resulting in no net growth in cell population. However, acute BRAF-V600E expression in PC Cl3 cells induces dedifferentiation and genomic instability, which, similarly to RAS, may facilitate the acquisition of secondary genetic or epigenetic events that may account for its aggressive properties (Mitsutake et al., 2005). In addition, the targeted expression of BRAF-V600E in thyroid cells of transgenic mice results in development of invasive PTC with poorly

differentiated foci that closely recapitulate the phenotype of BRAF-positive PTC in humans. The BRAF-V600E mice had a 30% decrease in survival at 5 months (Kim and Zhu, 2009 and references therein).
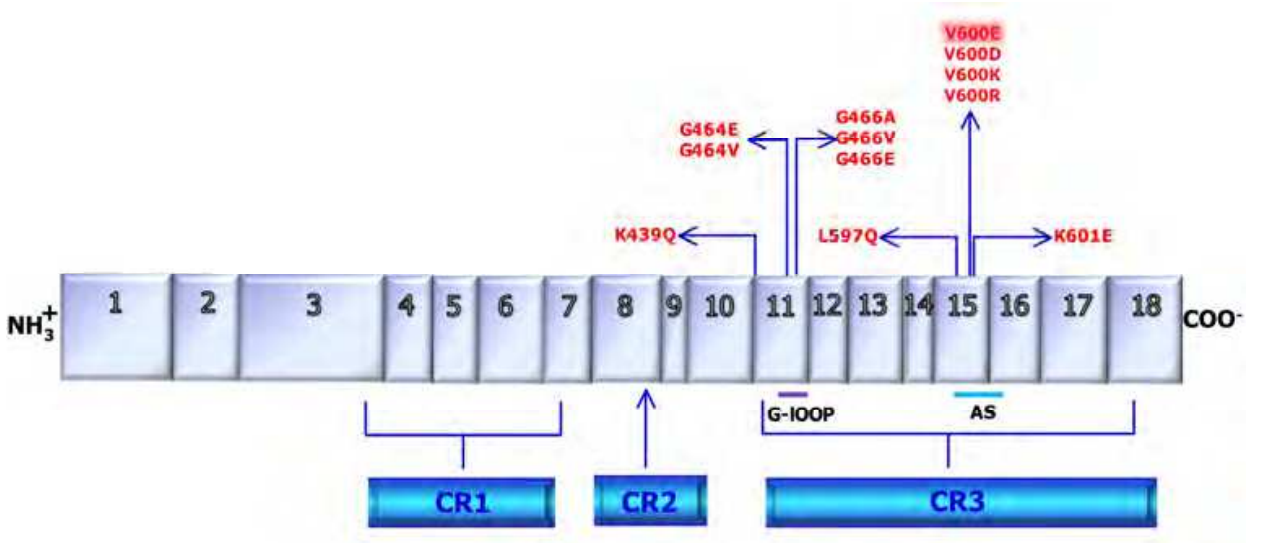

Fig. 5. BRAF mutations in thyroid cancer. The T1799A mutation accounts for about 90% of the more than 40 mutations identified in the BRAF gene so far. This mutation causes the V600E substitution in the BRAF protein that results in constitutive activation of the kinase and acquisition of oncogenic properties. Other BRAF mutations detected in human tumours are also reported. In thyroid cancer few other mutations that include the K601E have been reported.

3.1.3 The phosphatidylinositol 3-kinase (PI3K)/Akt pathway: Follicular thyroid carcinomas

Constitutive activation of the phosphatidylinositol-3-kinase (PI3K)/AKT signalling pathway plays a relevant in thyroid carcinogenesis (Bunney & Katan 2010). First, germline mutations of the tumor suppressor gene PTEN (phosphatase and tensin homologue deleted on chromosome 10) confer predisposition to Cowden disease, an autosomal dominant condition that causes hamartomatous neoplasms of the skin, gastrointestinal tract, thyroid, bones and predispose to CNS, breast and thyroid cancer (Hobert & Eng, 2009). Moreover, genetic alterations involving proteins within the PI3K/AKT pathway have been described in sporadic thyroid carcinomas, particularly in FTC and ATC (Ringel et al., 2001; García-Rostán et al., 2005; Ricarte-Filho et al., 2009). The reported alterations include genomic copy number gain or activating mutations of the gene encoding the catalytic subunit of PIK3CA, inactivating mutations, LOH or deletions of PTEN, and activating gain-of-function mutations in the AKT1 gene as discussed in detail below. The analysis of human thyroid cancer has also indicated that the PI3K/AKT pathway cooperates with MAPK signalling in the pathogenesis and progression of advanced or metastatic thyroid cancer.

PI3KCA mutations and amplifications

PI3Ks are a family of intracellular lipid kinases that generate the lipid second messenger PtdIns-3,4,5-P3 and PtdIns-3,4-P2. PI3K family members are grouped into three classes according to structure and substrate specificity (Engelman et al., 2006). Class I PI3Ks are

heterodimeric molecules composed of a catalytic subunit known as p110 and a regulatory subunit denoted p85, which contains two SH2 (Src homology) domains that allow interaction with phosphotyrosines on activated tyrosine kinase receptors. This results in recruitment of the protein to the plasma membrane and activation of the enzymatic activity. There are three variants of the p110 catalytic subunit designated p110α, β, or δ, expressed by separate genes (PIK3CA, PIK3CB, and PIK3CD, respectively). By contrast, there are five variants of the p85 regulatory subunit, designated p85α, p55α, p50α, p85β, or p55γ; the first three regulatory subunits represent splice variants of the same gene (PIK3R1), and the other two are encoded by different genes (PIK3R2 and PIK3R3, respectively) (Vanhaesebroeck, & Waterfield, 1999). So far a central role in cancer has been demonstrated only for class IA PI3Ks, which transduce signals downstream of oncogenic tyrosine kinase receptors. PIK3CA, encoding the class IA PI3K catalytic subunit p110α, is the only PI3K gene identified with common gain-of-function mutations and gene amplification in human cancer (Vogt et al., 2007). Most mutations are located in hot spot regions that include the helical and the kinase domains of the gene encoding p110α that result in a mutant protein that becomes independent of the p85 regulatory subunit thus promoting proliferation, invasiveness, resistance to apoptosis, and malignant transformation (Bader et al., 2006). In thyroid cancer, gene amplification/copy number gain of the PIK3CA gene located at 3q26.3 is detected in 12-13% of follicular adenoma, 5-14% PTC, 24-28% FTC and up to 42% of ATC, though ethnic variation between Middle Eastern, Western or Asian populations has been reported (Wang et al., 2007; Liu et al., 2008). In addition to increased gene copy number, recent studies have reported the presence of activating mutations of PIK3CA in primary thyroid cancer and cancer-derived cell lines. PIK3CA mutations are rare in primary well-differentiated PTC (0-3%), more frequent in well-differentiated FTC (6-13%) and common in ATC (5-21%) (García-Rostán et al., 2005). Importantly, PIK3CA mutations are particularly common in the metastatic lesions of patients with radioactive-iodine refractory disease. This finding suggests an exclusive role for oncogenic mutant PIK3CA in promoting progression from more differentiated to less differentiated cancer. At present, it is not known whether PIK3CA mutations or amplification are sufficient to cause thyroid cancer *in vivo*. Mutant PIK3CA alleles are transforming in MCF-10 breast cells *in vitro* and in the chorioallantoic membrane of the chicken *in vivo*. However, transgenic mouse models indicated that activated PIK3CA mutant is able to induce fully malignant cancer in the lung but not in the ovary (Engelman et al., 2008). Therefore, further studies will be required to fully characterize the role of this oncogene in thyroid cancer development and progression.

PTEN mutations and loss of expression

PTEN is a tumour suppressor gene localized to chromosome 10q23 (Li et al., 1997). PTEN has been shown to have protein and lipid phosphatase activity. PTEN can dephosphorylate the D3 position of PtdIns-3,4-P2 and PtdIns-3,4,5-P3, the lipid products of the PI3K, thus antagonizing signalling through this pathway. Reportedly, cells lacking PTEN function exhibit a marked increase in the intracellular levels of PtdIns-3,4,5-P3 and AKT activation. PTEN represents a pivotal regulator of critical cellular functions such as proliferation and survival. A large body of evidence indicate that PTEN functions as a tumour suppressor in thyroid cancer. Loss of PTEN is a frequent finding in sporadic tumours, through mutations and LOH, reduced transcription caused by gene promoter hypermethylation, reduced translation via microRNA (miR21) overexpression or increased protein degradation (Bunney & Katan, 2010). Mutations of PTEN are uncommon in sporadic thyroid tumours (2% PTC;

7%FTC and 14% ATC, respectively). Moreover, allelic losses of the PTEN locus at 10q23.3, though frequent in adenoma and FTC (up to 25%), are not coupled with mutations in the second allele. Conversely, thyroid carcinoma frequently shows decreased expression of PTEN, at both mRNA and protein levels in <40% of well-differentiated thyroid carcinomas and in most ATC, in many cases through methylation of the PTEN gene promoter. Expectedly, PTEN inactivation in human tumors has been associated with increased AKT activity. Yet, in transgenic mice loss of PTEN and the subsequent activation of the PI3K/AKT pathway causes goiter and follicular adenoma but it appears not to be sufficient for malignant transformation of thyroid cells (Kim and Zhu, 2009 and references therein).

AKT1 mutations and amplification

The AKT kinases represent the primary downstream mediators of the effects of the PI3K pathway, and play a central role in both normal and pathological signalling (Brazil et al., 2004). In mammalian cells AKT comprises three highly homologous members (>80% protein sequence identity) termed AKT1/PKBα, AKT2/PKBβ and AKT3/PKBγ, encoded by three different genes located on chromosomes 14q32, 19q13 and 1q43, respectively. AKT kinases share the same structural organization, containing an N-terminal pleckstrin homology (PH) domain, a central catalytic domain and a C-terminal regulatory region. The PH domain of AKT can bind specifically to D3-phosphorylated phosphoinositides with high affinity and mediates kinase activation (Brazil et al., 2004). Despite their sequence similarity however, AKT isoforms are functionally distinct, as suggested by the different phenotypes of the corresponding knock-out mice. Also the expression of AKT1, AKT2 and AKT3 apparently contribute to the different roles of AKT isoforms. AKT1 and AKT2 are the principal isoforms expressed in the thyroid gland (Vasko et al, 2004).

Combining all the data from the existing literature, it appears that activation of the PI3K/AKT pathway in thyroid cancer, as detemined by S473 phosphorylation, is frequent and is associated with aggressive disease. Active AKT is observed more frequently in patients with undifferentiated cancer (40-50% of PTC and FTC; <93% of ATC, respectively) (Wang et al., 2007; Santarpia et al., 2008). Different mechanisms that cause the increased AKT signalling observed in thyroid cancer cells have been proposed. First, gain-of–function mutations of two different AKT isoforms have been reported to occur in human cancer (Carpten et al., 2007; Davies et al., 2008). A unique mutation at nucleotide 49 of the gene encoding AKT1 that results in the substitution of a lysine for glutamic acid at the amino acid 17 (AKT1-E17K) within the PH has been recently discovered. The E17K substitution allows membrane recruitment of AKT1 independent of PtdIns binding, increases its activity, and confers to AKT1 the capability to transform fibroblasts *in vitro* and induce leukaemia in mice. More recently, a mutation homologous to the E17K in AKT1 has been identified also in the PH domain of AKT3 in malignant melanoma (Davies et al., 2008). In thyroid cancer, the presence of a heterozygote E17K mutation in the AKT1 gene was observed at a relatively high frequency (9/55, 16%) in metastatic lesions of advanced cancer but not in the corresponding primary tumours, which suggested that AKT1 mutations were acquired during tumour progression (Ricarte-Filho et al., 2009). AKT1 mutations were most common in metastasis of tall cell variant PTC (17%), Hürthle cell carcinoma (33%), and poorly differentiated PTC (19%). Conversely, no mutation in the genes encoding AKT2 and AKT3 has been reported in thyroid cancer so far. In addition to mutations, an increase in the gene copy number of AKT1 in FTC (8%) and ATC (<19%) and of AKT2 in FTC (<22%), respectively, has also been reported (Liu et al., 2008). It is not yet known whether amplified

AKT1 differs from mutated AKT1 in its capability to activate downstream signalling. Recent studies have suggested that cellular compartimentalization of activated AKT may be important in determining its cellular effects. In particular, it was proposed that nuclear localization of activated AKT1 promotes invasion and migration in thyroid cancer cells. In invasive FTC phospho-AKT localizes primarily to the nucleus, whereas in PTC, it localizes to the cytoplasm, except for the cells at the invasive edge or in metastatic regions where it is localized also in cell nuclei (Vasko et al. 2004).

Although aberrant activation of the PI3K pathway has been identified in most thyroid cancers, relatively few transgenic mice that model dysregulation of the PI3K/AKT pathway in cancer have been generated (Kim and Zhu, 2009 and references therein). Recently, a mouse strain, in which Cre-mediated recombination was used to delete Pten in the thyrocytes has been reported. Conditional loss of Pten in the thyroid gland renders the thyrocytes highly susceptible to neoplastic transformation through mechanisms that include increased thyrocyte proliferation. Pten mutant mice developed diffuse goiter characterized by enlarged follicles, in the presence of normal TSH and T4 hormone levels. Loss of Pten resulted in a significant increase in the thyrocyte proliferative index and increased cell density in the thyroid gland, which was more prominent in female mice. By 10 months of age, more than 60% of the mutant females developed follicular adenomas. However, in these mice complete loss of Pten was not sufficient to cause invasive tumors. Subsequent studies by the same group revealed that the in vivo proliferative response to chronic PI3K activation relied on the activation of the mammalian target of rapamycin (mTOR)/S6K1 axis, and that mTOR inhibition restored normal proliferation rates in Pten mutant mice. mTOR functions as a key effector of PI3K-generated proliferative signals by increasing the levels of cyclins D1 and D3 proteins through post-transcriptional mechanisms, and mTOR inhibition effectively restored normal D-type cyclin protein levels and normal proliferation rates in thyrocytes. Recently, double-mutant mice were generated by crossing a mouse strain carrying a KRAS-G12D allele with mice carrying the thyroid-specific floxed Pten. The concurrent activation of KRAS-G12D and PI3K in thyroid follicular cells led to aggressive, invasive and metastatic FTC, indicating that PI3K activation allowed to fully realize the oncogenic potential of KRAS. Interestingly, combined pharmacological inhibition of PI3K and MAPK completely inhibited the growth of double mutant cancer cells, providing a compelling rationale for the simultaneous targeting of these pathways in thyroid cancer. These results indicate that, at difference with genes involved in the MAPK pathways (i.e. BRAF) the constitutive activation of PI3K signalling is probably insufficient by itself to initiate the growth of a malignat thyroid cancer, since loss of PTEN results in follicular adenoma; conversely, aberrant PI3K signalling may facilitate progression and dedifferentiation of tumour cells.

3.1.4 Genetic alterations in transcription factors: Follicular thyroid carcinomas

The PAX8/PPARγ rearrangement

The PAX8-PPARγ rearrangement is a chromosomal translocation t(2:3)(q13;p25) that contributes to the development of thyroid cancers (Kroll et al., 2000). PAX8 (paired-box gene 8) encodes a transcription factor required for the development of thyroid follicular cell lineage and the regulation of thyroid-specific gene expression, whereas PPARγ (Peroxisome Proliferator-Activated Receptor-γ), encoded by the PPARG gene located on chromosome

3p25, is a member of the steroid nuclear hormone receptor superfamily. PPARγ plays a role in adipogenesis and insulin sensitization, cell-cycle control, inflammation, atherosclerosis, apoptosis and carcinogenesis through its influence on gene expression (Desvergne et al., 1999). The PAX8-PPARγ rearrangement was first identified in thyroid neoplasms with a cytogenetically detectable translocation t(2;3)(q13;p25) that generates a chimeric gene encoding the DNA-binding domain of PAX8 and domains A–F of PPARγ. The function of this rearranged protein is not entirely elucidated, but it appears that the fusion product contributes to malignant transformation by acting as a dominant negative on the transcriptional activity of wild-type PPARγ (Gregory Powell et al., 2004). PAX8-PPARγ rearrangements are present in follicular adenoma (up to 30%), FTC (25-63%), in follicular variants of PTC, and in Hurthle cell cancers, with the initial indication that it correlates with a vasculo-invasive phenotype (Kroll et al., 2000; Nikiforova et al., 2003). Conversely, the presence of a PAX8-PPARγ rearrangement in follicular variant of PTC is controversial and, to date, it has not been detected in PDTC and ATC (Nikiforova et al., 2004). Together, RAS and PAX8-PPARγ mutations are identified in approximately 80% of FTC (Nikiforova et al., 2004). However, the finding that both RAS and PAX8-PPARγ mutations may be rarely detected in the same tumor, suggests that these cancers develop through at least two different molecular pathways and the finding that the PAX8-PPARγ oncoprotein, like RAS, is also detected in a sub-group of follicular adenoma supports the existence of a stepwise transition from adenoma to carcinoma.

3.1.5 Genetic alterations of cell-cycle regulators

Alteration of the basic mechanisms that regulate cell cycle is a hallmark of cancer (Hanahan & Weinberg 2000). Cell cycle is regulated by the sequential activation of several classes of proteins (cyclins, cyclin-dependent kinases (CDKs), CDK inhibitors, the family of retinoblastoma susceptibility proteins (pRB), E2F transcription factors). The factors that promote progression into cell cycle are the G1 cyclins (i.e. cyclin D1, cyclin E1), CDKs, and E2Fs whereas the factors that regulate negatively the G1-to-S transition are the pRB, the two families of CDK inhibitors (INK4, CIP/KIP, respectively) and the tumor suppressor TP53. Cyclin–CDK complexes promote cell-cycle progression through phosphorylation-dependent inactivation of pRB, which in turn releases E2F transcription factors and allows entrance into S phase. Particularly important for cancer development are the G1/S and the G2/M transitions as determined by the frequent observation of aberrant activity of the molecules involved in these processes. See Figure 6 for a summary of the genetic alterations of cell-cycle regulators observed in thyroid cancer.A reasonable anticipation is that the growth of well-differentiated thyroid carcinoma is relatively low compared with PDTC and ATC and that the altered expression and/or activity of cell-cycle regulators determine these differences in growth. Accordingly, the MIB-1 index is 1–3% in WDTC, 6–7% in PDTC and 14–52% in ATC (Katoh et al., 1995). A high-labeling index, as seen in ATC and poorly differentiated thyroid patients, correlated with persistent disease or death (Kjellman et al., 2003). Expectedly, as is the case with other common human carcinomas, a series of multiple alterations in cell cycle control-related gene products such as up-regulation of CDKs, down-regulation of CDK inhibitors or both, frequently contribute to the pathogenesis of thyroid cancer (Kondo et al. 2006). Cyclin D1 (which is encoded by CCND1 on chromosome 11q13) and cyclin E1 (which is encoded by CCNE1 on chromosome 19q12) are overexpressed in thyroid cancer. Overexpression of cyclin D1 is observed in approximately 30% of FTC and
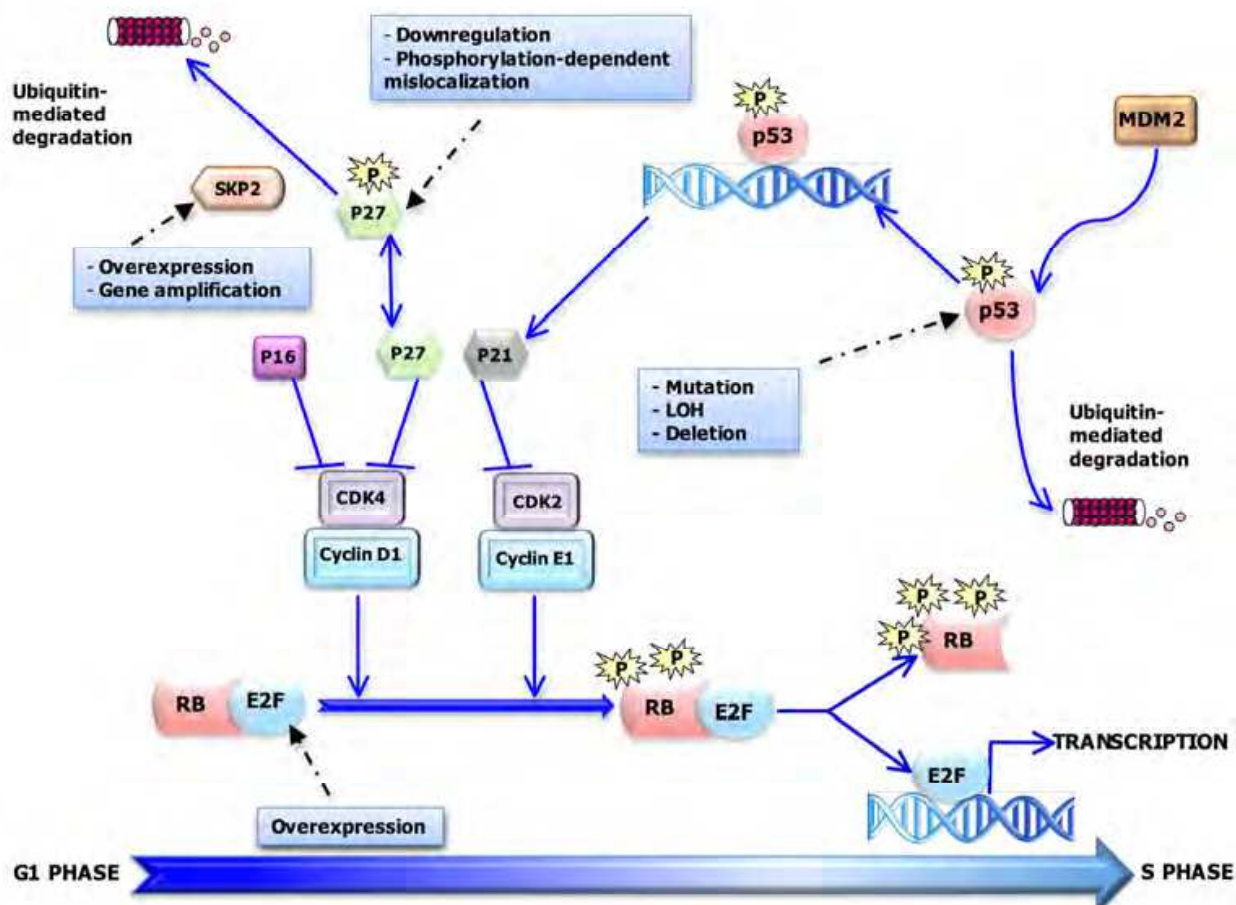
Fig. 6. Molecular alterations of cell-cycle regulators in thyroid cancer. The cyclin D1/CDK4 and cyclin E1/CDK2 cooperate to control the G1 to S phase transition through the phosphorylation of retinoblastoma protein (pRB). Hypophosphorylated pRB functions as a repressor of E2F transcription factors; conversely, inactivation of pRB through phosphorylation allows E2F activity. In particular, E2F activates the transcription of genes that are involved in the G1 to S phase transition, such as DNA polymerase and thymidine kinase. The CDK inhibitors p16INK4A, p21CIP1 and p27KIP1 impair the activity of cyclin/CDK complexes, thus preventing phosphorylation of pRB. Therefore, cyclins and CDKs function as oncogenes whereas CDK inhibitors function as tumour suppressors. The tumour suppressor TP53 induces cell-cycle arrest by up-regulating p21CIP1, another CDK inhibitor. The function of TP53, in turn, is controlled by negative regulators, including MDM2, which targets TP53 for ubiquitin-mediated degradation, constituting a feedback loop that maintains a low concentration of TP53 in the cells.

76% and PTC, respectively, having been correlated with metastatic dissemination of PTC (Lazzareschi et al., 1998). Similarly, cyclin E1 is overexpressed in a large number of thyroid carcinomas (Lazzareschi et al., 1998). At difference with other cancer types that show amplification or inversion of the locus containing CCND1, in thyroid cancers these genes are neither amplified nor rearranged (Lazzareschi et al., 1998). Therefore, overexpression of cyclins in thyroid tumours is a secondary effect that is induced by other genetic aberrations, such as RAS mutations or RET/PTC rearrangements. CDK inhibitors – both INK4 and CIP/KIP proteins - are commonly down-regulated in thyroid malignancies. Point mutations of CDKN2A on chromosome 9p21, which encodes p16INK4A, though common findings in glioma and melanoma, are rare in thyroid tumours. Alternatively, LOH in the chromosomal region spanning the CDKN2A locus is associated with FTC (27%) and ATC (50%), and methylation of 5′ CpG islands of CDKN2A promoter is detected in 30% of thyroid neoplasms (Kondo et al., 2006 and references therein). As to the CIP/KIP proteins, normal and hyperplastic follicular cells show strong immunoreactivity for p27KIP1 (encoded by CDKN1B on chromosome 12p13), whereas its expression is significantly reduced in PTC, FTC and ATC (Erickson et al, 2000). P27 down-regulation in thyroid cancer depends on the over-expression of the ubiquitin-protein ligase Skp2, which is amplified in several thyroid tumors. Skp2 expression correlates with p27KIP1 down-regulation; forced expression of Skp2 circumvented serum-dependency and contact inhibition in Skp2-negative cells by promoting p27 degradation; and finally, the suppression of Skp2 expression drastically reduces proliferation of thyroid cancer cells. On the other hand, p27KIP1 that normally resides in the nucleus, is frequently inactivated by mislocalizion to the cytoplasm, a mechanism linked to AKT-dependent phosphorylation. P21CIP1, another CIP/KIP inhibitor encoded by CDKN1A gene on chromosome 6p21, is expressed in 40% of well-differentiated PTC 7% of PDTC, and not in ATC. On average, 10-13% of thyroid malignancies harbour CDKN1A deletions on chromosome 6p21 (Shi et al., 1996). The expression pattern of pRB in benign and malignant thyroid lesions is controversial. Although one group has reported the presence of inactivating mutation in the gene encoding pRB1 (located on chromosome 13q14) in 55% of thyroid carcinomas this has not been confirmed by other investigators. The main targets of pRB are represented by the E2F transcription factors, which consists of six members: E2F1–E2F6. E2F-regulated genes are repressed by pRB proteins; such a repression is alleviated by CDK-dependent phosphorylation of pRB. E2F1, but not other members of this family, is up-regulated in 35–89% of WDTC, 34% of PDTC and 67% of ATC (Volante et al., 2002). As part of the cell cycle surveillance system, the G2 spindle checkpoint protects the cell from genomic instability. Entry into mitosis is blocked by the G2 checkpoint that ensures that chromosomes are not segregated to daughter cells when DNA is damaged. Thyroid cell transformation is accompanied by the overexpression of a cell proliferation/genetic instability-related gene cluster that includes Polo-like kinase 1 (PLK1), a protein kinases involved in in several G2- and M-phase–related events such as centrosome maturation, proper spindle formation, cyclin B/Cdk1 activation, anaphase-promoting complex/cyclosome (APC/C) activation, chromosome segregation, and cytokinesis (Salvatore et al., 2006). ATC, but not normal thyroid, cells are dependent on PLK1 for survival. RNAi-mediated PLK1 knock-down caused mitotic arrest associated with 4N DNA content and massive mitotic cell death (Nappi et al., 2009). Other alterations implicated in the G2/M transition include overexpression of the Aurora A-C kinases in ATC cell lines and tumors (Sorrentino et al. 2005) and the mitotic spindle assembly checkpoint genes hBUB1, hBUBR1 and hMAD2 (Wada et al. 2008).

ANIMAL MODELS OF THYROID CANCER

	MOUSE LINE	STRAIN	PATHOLOGY	METASTASIS	THYROID FUNCTION
PTC	RET/PTC1	C557BL/6J	22% WITH PTC	NO	NA
	RET/PTC1	FVB/N	100% WITH MULTIFOCAL PTC	NO	NORMAL/HYPOTHYROIDISM
	RET/PTC3	C3H/He	31% WITH PTC BY 3 MONTHS	LYMPH NODES	NA
	BRAF^{V600E} (Tg-BRAF2)	FVB/N	93% WITH PTC BY 3 MONTHS	LYMPH NODES	TSH INCREASED
	BRAF^{V600E} (Tg-BRAF3)	FVB/N	25% WITH PTC AT 3 MONTHS	NO	TSH INCREASED
	TRK-T1	B6C3F1	23% WITH PTC-LIKE CANCER	NO	NA
	H-Ras^{G12V}	MIXED	3 OUT OF 4 MICE DEVELOPED PTC	UNCLEAR	NORMAL T4 LEVEL
	Prkar1a$^{D2/+}$	MIXED	95% WITH PTC-LIKE CANCER	NO	NA
FTC	K-Ras^{G12V}	C57BL/6J, DBA/2	2% WITH FTC	NO	NORMAL
	Pten -/-	FVB/N x 129Sv	FTC IN FEMALES	NO	NORMAL TSH AND T4 LEVELS
	N-Ras^{Glu61Lys}	FOUNDERS x C57BL/6J	11% ADENOMA, 29,5% FTC/MIXED	LIVER, LUNG, BONE	ELEVATED TSH
	K-Ras^{G12D} Pten -/-	129SV	100% FTC WITH LOCAL INVASION	LUNG	LOW TSH/HIGH T4
	TRb$^{PV/PV}$	C57BL/6J x 129Sv	100% FTC	LUNG	ELEVATED TSH, T3 AND T4
	1b-adrenergic receptor	FOUNDERS x C57BL/6J	3 OUT OF 6 LINES WITH GOITER AND FTC	LUNG	ELEVATED T4
	Rap1b^{G12V}	FVB (Taconic)	FTC AFTER TREATMENT WITH A GOITROGEN	NO	NORMAL

Table 2. Animal models of thyroid carcinomas (from Kim and Zhu, 2009, modified).

3.1.6 Genetic alterations in anaplastic thyroid carcinomas

The tumour-suppressor gene TP53

Most of the mutations discussed so far are mainly found in differentiated thyroid cancers and are believed not to be sufficient by themselves to trigger the progression to PDTC and ATC. By contrast, mutations of TP53, a tumor suppressor gene located on chromosome 17, are common features of PDTC or ATC, and could be responsible for the loss of differentiation observed during tumor progression. In thyroid cancers, TP53 mutations occur in 17–38% of PDTC and 67–88% of ATC, respectively, and only in isolated cases of differentiated PTC and FTC (Nikiforov, 2008 and references therein). TP53 is a key gatekeeper that plays a role in cell cycle regulation, apoptosis, genomic stability, and inhibition of angiogenesis. In its anti-cancer role, TP53 can induce growth arrest by holding the cell cycle at the G1/S or G2/M points following DNA damage recognition, which prevents replication of cells with damaged DNA and allows the DNA repair proteins to have time to fix the damage and resume the cell cycle. Alternatively, TP53 can initiate the programmed cell death if DNA damage proves to be irreparable Activation of wild-type TP53 can lead to G1 cell-cycle arrest through transcriptional induction of the CDK inhibitor p21CIP1, or apoptotic cell death by activating transcription of pro-apoptotic molecules such as BAX and FAS. Loss-of- function mutations of TP53 impair its transcriptional activity and induce genomic instability, owing to weakened DNA repair systems, and subsequent cancer progression.

β-catenin mutations

β-catenin, encoded by the CTNNB1 gene on chromosome 3p22–21.3, plays a role in cell adhesion and transcription. In normal cells, most β-catenin protein is bound to E-cadherin

(encoded by the CDH1 gene on chromosome 16q22) in the cytoplasmic portion of adherens junctions, thus fulfilling an essential role in cell adhesion. This binding sequesters β-catenin from the nucleus and restrains its growth-promoting role. β-catenin is a critical regulator of cell proliferation induced by Wnt signalling, promoting transcription of cyclin D1 and MYC (Cadigan & Peifer, 2009). The cellular abundance of β-catenin is finely modulated through proteasomal degradation. This process occurs through the action of a multicomponent complex that includes APC - encoded by the APC gene inactivated in familial adenomatous polyposis –the scaffold protein Axin and the Glycogen Synthase Kinase-3 (GSK), which phosphorylates β-catenin and targets it for polyubiquitination and degradation. Activation of the Wnt pathway inhibits GSK-3-dependent phosphorylation of β-catenin as well as its subsequent proteosomal degradation, allowing β-catenin to translocate to the nucleus and function as a transcriptional effector of Wnt. In cancer cells the growth-promoting activity of β-catenin is enhanced either by reducing its binding to E-cadherin (e.g. due to decreased CDH1 expression), or when APC-Axin-GSK3β-mediated degradation of β-catenin is defective due to inactivating mutations of APC and/or CTNNB1 or to overactive Wnt signalling. Such mutations disrupt phosphorylation sites of β-catenin and lead to protein stabilization. Mutations and abnormal nuclear localization of β-catenin have been observed, along with overexpression of its target genes c-Myc and cyclin D1, in thyroid malignancies (Ishigaki et al., 2002). Although increased levels of cytoplasmic β-catenin are observed in most thyroid cancer cells, mutations of β-catenin that lead to nuclear localization of the protein are limited to PDTC and ATC suggesting a role in tumor progression (Garcia-Rostan et al., 2001). On the other hand, E-cadherin is highly expressed in normal thyroid and benign adenoma but its expression is consistently decreased in cancer, especially in recurrent or metastatic carcinomas. Mutations of CDH1 are infrequent in undifferentiated cancer; conversely loss of E-cadherin is due to aberrant methylation of the CDH1 promoter (Kato et al., 2002). Another observation that supports a role of the APC–β-catenin pathway in the development of thyroid cancer is that familial adenomatous polyposis (FAP) and its variant, Gardner syndrome, which confers a markedly increased risk of development of PTC are caused by germline mutations in the APC gene. However, it appears that the aberrant nuclear localization of β-catenin observed in thyroid carcinoma is more likely induced by CTNNB1 mutations rather than APC mutations.

3.1.7 Genetic alterations of microRNA in thyroid cancer

MicroRNAs (miRs) are a class of 19–23 nucleotide-long non-coding RNAs that negatively regulate gene expression through either the inhibition of mRNA translation or the induction of its degradation (Ambros 2004). MiRs are transcribed by RNA polymerase II in the nucleus, are transported into the cytoplasm by the Exportin system where they are incorporated into the RISC, thus acquiring the ability to bind to the 3' untranslated region (UTR) of the target mRNAs causing mRNA degradation or the block of translation (Ambros 2004, Bartel 2004). At present it is estimated that there are 300–1000 microRNAs, each of which may bind to several hundred mRNA targets. MiRs are involved in a wide range of basic processes such as cell proliferation, development and apoptosis (Bartel 2004). MiRs are abnormally expressed in many types of human cancer and can act as oncogenes or tumor suppressor genes or, in some cases, can perform both functions (Calin & Croce 2006,). Recent studies have shown that miRs may also contribute to onset and/progression of thyroid malignancies. Most studies have focused on the analysis of miR expression profile

of PTC by 'miRCHIP' microarray. Several miRs including miR-221, -222, -146, -21, -155, -181a, and -181b have been shown to be up-regulated in PTC compared with the normal thyroid (Pallante et al., 2005). In particular miR-221, -222, and -181b have been proposed to represent a signature for PTC. MiR-221 and -222 represent the most consistently up-regulated miRs in PTC. They are very similar in sequence, clustered on chromosome X, and are likely transcribed as polycistronic transcripts (Ciafrè et al. 2005). Adoptive expression of miR-221/222 significantly modifies proliferation of thyrocytes, increasing G1- to S-phase transition through the reduction of p27KIP1 protein levels (Visone et al. 2007a). However, the mechanism by which these miRs are upregulated in PTC is still under investigation, since no gene amplification or changes in the methylation status has yet been found. Although most of the studies conducted so far have focused on miR expression in PTC, Nikiforova et al. (2008) reported on a signature specifically associated with follicular adenoma and FTC. The most highly up-regulated miRs in conventional FTC were miR-187, -224, -155, -222, and -221, and those in oncocytic variants were miR-187, -221, -339, -183, -222, and -197. In a different study, four miRs that are differentially expressed between FTC and adenoma (miR-192, -197, -328, and -346) have been identified (Weber et al. 2006). Inhibition of miR-197 and - 346 in human thyroid cancer cells (FTC133) caused growth arrest (Weber et al. 2006). MiR-21 targets E2F and inhibits PTEN. Recent data indicate that specific miRs are associated with different histological types of thyroid. MiR-187 is expressed at high levels in PTC harboring RET/PTC rearrangements whereas miR-221 and -222 are found at the highest level in BRAF- and RAS-positive PTC and those with no known mutations. Conversely, RAS-positive PTC expresses high amount of miR-146. In ATC samples and cell lines the miR-17–92 cluster containing seven miRs as well as miR-106a and -106b are overexpressed (Takakura et al. 2008). Antisense inhibition of miRs 17-3p, -17-5p, and -19a causes cell cycle arrest, and suggests an oncogenic role for these miRs. MiR-19a and -19b in the cluster have PTEN as a target, and miR-106a and -106b have E2F1 as a target, thus suggesting that there are multiple potential therapeutic targets in the miR-17–92 cluster (Takakura et al. 2008). On the other hand, four miRs (-30d, -125b, -26a, and 30a-5p) have been shown to be under-expressed in ATC but not in PTC (Visone et al. 2007b). MiR-26a and -125b target HMGA1 and HMGA2, two proteins causally involved in thyroid cell transformation. In addition, miR-138 that targets hTERT is reduced in ATC (Visone et al., 2007b). In conclusion, the studies of miRs expression and function indicate that each of the three principal types of thyroid cancer has several distinct miRs already and hold promise to improve the evaluation and management of these tumors.

4. Targeted therapy of thyroid cancer

The current treatment of patients with differentiated thyroid cancer includes surgery, radioactive iodine administration and thyroid hormone suppression therapy and is, in most cases, effective. Accordingly, survival rates for patients with local differentiated thyroid carcinoma are excellent. By contrast, treatment of patients with advanced thyroid cancer continues to represent a significant challenge for clinical oncologists. These patients are not responsive to standard treatment and require additional therapies. However, the efficacy of cytotoxic chemotherapy is poor and that of external beam radiation has not been established yet. It is likely that this scenario has just started to change because of the introduction of

targeted therapies - especially tyrosine kinase inhibitors - for the treatment of advanced thyroid cancer (Santoro & Carlomagno, 2006). Tyrosine kinase inhibitors cause tumor shrinkage and/or disease stabilization. The rationale for the development of specific inhibitors of the oncogenes that initiate cancer is based on the hypothesis denoted the "oncogene addiction" (Weinstein &, Joe, 2008). According to this hypothesis, the initiating genetic alteration that hits a normal cell and starts the transformation process becomes essential for the survival of cancer cells. Thus, inhibition of the oncogene that initiates a certain cancer is expected to lead to either tumor stabilization or regression. For this reason, a lot of interest arose in the therapeutic potential of kinase inhibitors for thyroid cancer patients. The prevalence of activating BRAF mutations, RET/PTC rearrangements and RAS mutations that is reflected into consequent downstream activation of ERKs, suggests that activation of the MAP kinase pathway may be an obligatory step in the transformation of thyrocytes. Therefore, such dependency may represent a potential Achilles heel of cancer cells. Since thyroid cancer cells are apparently "addicted" to aberrant MAP kinase signalling, several small molecules that target this pathway are currently being developed (Sherman, 2009). Several lines of evidence suggest that RET/PTC can be a good target. The quinazoline low molecular weight tyrosine kinase inhibitor ZD6474 (Vandetanib), a potent inhibitor of the VEGF receptor-2 (flk-1/KDR), has also been shown to inhibit the enzymatic and transforming activity of RET/PTC oncoproteins and to block the growth of RET/PTC3-induced tumours in nude mice (Carlomagno et al., 2002). Multiple phase II clinical trials testing the efficacy of ZD6474 in patients with metastatic medullary thyroid cancer, as well as metastatic papillary cancer are currently underway. In the case of patients with metastatic familial medullary thyroid carcinoma one of these clinical trials demonstrated partial response in 17% of patients and stable disease in another 33% (Sherman, 2008a).

BRAF represents another valuable therapeutic candidate for treatment of thyroid cancer due to the high frequency of BRAF mutation in thyroid tumors and its association with tumor dedifferentiation and resistance to the conventional radioiodine therapy. The biaryl urea Sorafenib (BAY 43-9006) is a potent inhibitor of BRAF, VEGFR and RET (Wilhelm et al., 2004). Sorafenib has shown cytostatic effects in thyroid tumor cells lines, both with and without the presence of BRAF mutations (Salvatore et al., 2006). In xenografts, daily administration of sorafenib inhibits phospho-MEK activity, attenuates tumour growth, and reduces Ki67/MIB-1 staining. Sorafenib received approval from the US Food and Drug Administration for the treatment of metastatic renal cancer, a malignancy where BRAF mutations have not been observed. In this case, it is believed that the clinical efficacy of sorafenib may derive more from its anti-VEGF activity than from BRAF block. Data from multiple clinical studies for the treatment of advanced thyroid cancer with sorafenib have been reported (Gupta-Abramson et al., 2008; Hoftijzer et al., 2009; Kloos et al., 2009). Three phase II studies have been conducted to determine the efficacy of sorafenib in advanced thyroid carcinomas of follicular origin. Despite its promising preclinical properties, the preliminary efficacy data for sorafenib in patients with thyroid cancer appear modest. This drug was shown to have a partial response in some patients with progressive PTC. Currently, phase II clinical trials are underway using BAY 43-9006 in the treatment of ATC and metastatic MTC. Preliminary results of the trial in patients with progressive PTC have shown minimal or partial response in some patients. However, several second-generation small molecule inhibitors of BRAF and MEK that exhibit *in vitro* activity exceeding that of

sorafenib are currently being investigated. Presumably, these and other emerging RAF inhibitors may provide a more robust effect against MAP kinase activity in clinical trials.

Another strategy to block growth of thyroid tumor is through the inhibition of angiogenesis (Ferrara & Kerbel, 2005). This results in reduced delivery of oxygen and nutrients to tumor cells and a reduced removal of waste and CO2, which ultimately compromises cell viability. VEGF is a stimulator of angiogenesis that substantially contributes to tumor progression. AMG 706 is an ATP-competitive inhibitor of VEGFR1, VEGFR2 and VEGFR3 that inhibits VEGF-induced cell proliferation and vascular permeability, thus inducing tumour regression *in vivo* (Polverino et al. 2006). AMG 706 has shown encouraging anti-tumour activity in a subset of patients with iodine-refractory metastatic thyroid cancer in a phase I study.

Axitinib is an oral tyrosine kinase inhibitor that effectively blocks VEGFR1, VEGFR2 and VEGFR3 at subnanomolar concentrations. As AMG 706, it also appears to inhibit c-KIT and PDGFRβ. A multicenter phase II study examined the efficacy of axitinib in advanced or metastatic thyroid carcinoma (Cohen et al., 2008a). Among the 45 evaluable patients, 30% experienced a partial response and 38% presented stable disease lasting more than 16 weeks, yielding an objective response rate of 30% and a disease control rate of 68%.

Motesanib diphosphate is an oral inhibitor that blocks VEGFRs at nanomolar concentrations (VEGFR1 IC50 of 2 nM; VEGFR2 IC50 of 3 nM; VEGFR3 IC50 of 6 nM) (Sherman et al., 2008b). Moreover, it appears to inhibit c-KIT (IC50 of 8 nM), PDGFRβ (IC50 of 84 nM) and RET (IC50 of 59 nM) either wild type or mutated. A multicenter phase II study examined the efficacy of motesanib in locally advanced or metastatic, radioiodine-resistant differentiated thyroid cancer (Sherman et al., 2008b; Schlumberger et al., 2009). The study yielded an objective response rate of 14% and a disease control rate of 81%. The median estimate of progression-free survival was 40 weeks.

Finally, sunitinib is an oral inhibitor that inhibits VEGFR1 (IC50 of 2 nM), VEGFR2 (IC50 of 9 nM), VEGFR3 (IC50 of 17 nM), RET (IC50 of 41 nM), RET/PTCs (for RET/PTC3 IC50 of 224 nM) and PDRGRβ (IC50of 2nM) (Kim et al., 2006). In an initial phase II study 43 patients with metastatic, iodine-refractory thyroid carcinoma of all histological sub-types were enrolled (Cohen et al., 2008b). Partial response was observed in 13% of patients, stable disease in 68%, and progressive disease in 10%, yielding an objective response rate of 13% and a disease control rate of 81%. As the results of additional ongoing clinical trials are expected to be available in the near future, it is expected to reach a more precise assessment of the role of such molecular inhibitors, administered alone or in combination, in the therapy of thyroid cancer.

5. Conclusions

In conclusion, our increasing understanding of the biology of thyroid follicular cancer is leading to the development of novel and promising therapies. Tumour-initiating events have been identified in a high proportion of the most frequent types of thyroid cancer - PTC, FTC, and ATC. All the genetic alterations identified so far converge in few signalling cascades – the RTK/RAS, the BRAF/MAPK and the PI3K/AKT pathways, respectively. This provides a strong basis for the development of novel gene-based diagnostic, prognostic, and therapeutic strategies. In fact, the treatment of advanced thyroid cancer is changing dramatically following the development of kinase inhibitors. It remains to be

determined whether combinations of targeted therapies with chemotherapy or radiotherapy will improve response rates. The results of the on-going clinical trials, as well as the new agents in development, will likely contribute to improve the lives of patients with advanced thyroid cancer.

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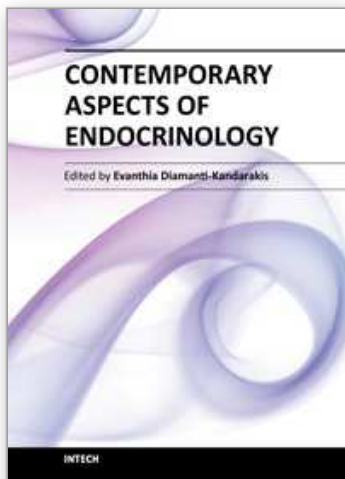
Contemporary Aspects of Endocrinology

Edited by Dr. Evanthia Diamanti-Kandarakis

This book aims to provide readers with a general as well as an advanced overview of the key trends in endocrine disorders. While covering a variety of topics ranging from thyroid carcinogenesis and pituitary adenomas to adrenal tumors and metabolic bone disease, this book also focuses on more specific issues not yet fully elucidated (e.g. the molecular pathways involved in thyrotropin beta gene regulation or monogenic phosphate balance disorders). Readers of different fields and background will have the opportunity to update their knowledge and more importantly to clarify areas of uncertainty and controversies in several topics of endocrine disorders.

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