New insights in thyroid follicular cell biology and its impact in thyroid cancer therapy

Garcilaso Riesco-Eizaguirre\textsuperscript{1,2} and Pilar Santisteban\textsuperscript{1}

\textsuperscript{1}Instituto de Investigaciones Biomédicas ‘Alberto Sols’, Consejo Superior de Investigaciones Científicas y Universidad Autónoma de Madrid (CSIC-UAM), 28029 Madrid, Spain
\textsuperscript{2}Servicio de Endocrinología y Nutrición, Hospital Universitario La Paz, Madrid, Spain

(Correspondence should be addressed to P Santisteban; Email: psantisteban@iib.uam.es)

Abstract

Well-differentiated thyroid cancer has in general terms a very good outcome. It has a very slow growth rate and, although it metastasises at a relatively high frequency, it has very high survival rates. Whereas the prevalence of nodular thyroid disease worldwide is high, malignant conversion from benign thyroid nodules is rare. Treatment of thyroid cancer is usually successful, but we still do not have effective therapies for patients with invasive or metastatic thyroid cancer if the disease does not concentrate radioiodine and it is not surgically resectable. On the other hand, from the same thyroid cell, one of the most aggressive human tumours can arise – undifferentiated or anaplastic thyroid carcinoma – leading to death in a few months. What features of this malignancy account for such paradoxical behaviour? The most common type of thyroid cancer – papillary thyroid carcinoma – stands out among solid tumours because many of the tumour-initiating events have been identified. All of them function in a single pathway – the RTK/RAS/RAF/MAPK pathway – and obey an ‘exclusivity principle’: one and only one component of the pathway is mutated in a single tumour. This highlights the requirement of this signal transduction pathway for the transformation to thyroid cancer and paves the way to targeted therapies against a tumour with a mutation in a known gene or any gene upstream of the target. However, it is also interesting to underscore the differences among the tumours arising from the different mutations. Studies \textit{in vitro} and \textit{in vivo}, including genomic profiling and genetically engineered mouse models, have clearly shown that each oncoprotein exerts its own oncogenic drive, conferring a distinct biological behaviour on thyroid tumours. In this review, we attempt to summarise the most recent advances in thyroid follicular cell-derived cancers research and their potential clinical impact that may change the management of thyroid cancer in the near future.

\textit{Endocrine-Related Cancer} (2007) 14 957–977

The disease

Thyroid cancer incidence has increased significantly during the past decades (Davies & Welch 2006) and it has become one of the ten leading cancer types in females, accounting for 22 590 new cases per year in the United States; it is more frequent than ovarian, urinary bladder or pancreas cancer (Jemal \textit{et al}. 2007). As diagnostic techniques for thyroid cancer have become more sensitive, particularly with the advent of ultrasound and fine needle aspiration (FNAS), the increasing incidence of thyroid cancer is predominantly due to the increased detection of small papillary cancers or microcarcinomas. Additionally, the 5-year relative survival rates for thyroid cancer have increased significantly from 93\% in 1983–1985 to 97\% in 1995–2001, probably due to the same reason (Jemal \textit{et al}. 2007). In this review, we will focus mainly on the thyroid follicular cell-derived cancers.

Thyroid cancer management has not changed substantially during the past decades. Treatment is based on total thyroidectomy, ablative doses of radioiodine and suppressive treatment. The follow-up is based on measuring serum thyroglobulin (Tg) and imaging with radioiodine scans. Since among clinicians there is a tendency to overtreating patients with thyroid cancer, recently new proposals to change this...
algorithm have arisen (Pacini et al. 2006). Most likely, the 2.4-fold increase in incidence of papillary microcarcinoma has also something to do with this. It is a fact that a more accurate prognostic stratification that improves thyroid cancer management is lacking (Udelsman & Shaha 2005).

There are four main critical challenges that remain unsolved in thyroid cancer management concerning diagnosis, prognosis and therapeutic management. First, 15–20% of the FNAS are inconclusive or cannot discriminate between follicular adenoma and carcinoma. This means that the patient needs to undergo partial or total thyroidectomy only for diagnostic purposes. Moreover, a sequential thyroidectomy has to be performed in the case of follicular thyroid carcinoma (FTC). Second, it has been estimated that about 20% of the patients with well-differentiated thyroid carcinoma will develop a local or distant recurrence and 1% will die (Schlumberger & Pacini 2003). Identifying these high-risk patients at the time of diagnosis through well-established prognostic factors can help to ascertain the most appropriate treatment and follow-up for these patients. Although several prognostic scoring systems, like the tumour node metastasis classification (AJCC 2002), have been developed for thyroid cancer, unfortunately there is still a debate on the definition and best management of low- versus high-risk patients, and each centre has its own protocols (Schlumberger & Pacini 2003). Third, there is still no treatment for one of the most intriguing situations that an endocrinologist may have to face: patients with elevated serum Tg and negative 131I scans. This is challenging for thyroid cancer management, as anatomical localisation of recurrences cannot be assessed and treatment with ablative doses of 131I is not effective, predicting a poorer outcome. Fourth, anaplastic thyroid carcinoma, although rare, is extremely aggressive, leading to death in 100% of the cases in a few months.

It is worth pointing out that about 500–600 million people suffer nodular goitre worldwide, and 4–6% of American adults are goitrous despite normal iodine intake (Freitas 2000). Thyrocyte proliferation leading to single nodule or multinodular goitre is diagnosed every day, even in children, and thyroid nodules are detected in more than 50% of autopsies (Derwahl & Studer 2000). These figures are quite high and, although malignancies are only present in 5% of all the thyroid nodules, there is a need for accurate and sensitive techniques that will help clinicians to discriminate among the vast amount of thyroid nodules diagnosed every day.

Beyond this clinical pathological picture, thyroid cancer biology has unique characteristics compared with other human tumours that we must bear in mind. First, there is a surprisingly low frequency of malignant conversions, given the high incidence of benign tumours in the normal population. Moreover, no premalignant carcinomas have been identified for the most frequent type of thyroid cancer, papillary thyroid carcinoma (PTC). Second, the very slow growth rates of well-differentiated thyroid carcinomas independently of the evaluation method used, contrasts with the much faster growth rates of well-differentiated breast, lung, colon and stomach adenocarcinomas (Soares & Sobrinho-Simoes 1994, Katoh et al. 1995, Sreelekha et al. 2000). Third, well-differentiated thyroid carcinomas show little or no apoptosis (Moore et al. 1998, Yoshida et al. 1999, Sreelekha et al. 2000). Fourth, even though survival rates are high, well-differentiated thyroid carcinomas metastasise at a relatively high frequency (Schlumberger & Pacini 2003). The purpose of this review is to summarise the most relevant advances in thyroid cancer research and how these new insights will impact on therapeutic options in the near future.

The genetics: cancer genes and the pathways they control

Cancer is, in essence, a genetic disease. Many genes that have a causal role in cancer have been discovered and the delineation of the pathways through which they act characterised (Vogelstein & Kinzler 2004). Focusing in pathways rather than individual genes is based on the fact that there are always a variety of genes that, when altered, lead to similar phenotypes. This concept is very familiar to genetists and as there are many fewer pathways than cancer genes, application of this concept to cancer has been established in the past years by elucidation of biochemical functions of the altered cancer genes, either in cell culture systems or in mice.

The RTK / RAS / RAF / MAPK pathway

The most studied pathway involved in thyroid tumorigenesis is the RTK/RAS/BRADF/MAP kinase pathway, which seems to be essential for the development of PTC (Fig. 1). On the contrary, this pathway seems to play a more limited role in FTC. Accordingly, the main genetic events discovered so far related to PTC play important roles in this pathway and, as in many other cancers, these genetic events do not overlap, which highlights the requirement of this signalling system for transformation to PTC. However, although all the aforementioned
genetic events activate this pathway, notable differences can be found among them.

MAPKs regulate critical cellular functions required for homeostasis such as the expression of cytokines and proteases, cell cycle progression, cell adherence, motility and metabolism. MAPKs therefore influence cell proliferation, differentiation, survival, apoptosis and development; and not surprisingly, they also control the growth and survival of a broad spectrum of human tumours. Most studies exploring the effects of oncogenes have been performed in cells in whose growth is negatively regulated by cAMP. Thyroid cells are an exception, since they are dependent on the presence of thyrotrophin (TSH) for growth, primarily through activation of adenylyl cyclase, cAMP generation and stimulation of protein kinase A activity (Medina & Santisteban 2000). Furthermore, in contrast with other cancers (Dorsam & Gutkind 2007), activating mutations of the main G-protein-coupled receptor of the thyroid, the TSH receptor or its Gsa subunit, do not lead to malignancy, but to toxic benign nodules. This confers a unique trait on thyroid cells that, as we will see below, may serve as a protective mechanism for tumour initiation in the thyroid.

**RAS family genes**

RAS oncogenes were the first to be associated with thyroid cancer. The RAS protooncogenes encode 21 kDa G-proteins, which transduce signals from a wide variety of growth factor receptors, particularly those of the tyrosine kinase family. Point mutations affecting the guanosine triphosphate (GTP)-binding domain (codon 12/13) or the GTPase domain (codon 61) result in the replacement of specific amino acid residues that lock p21RAS in the active form, resulting in constitutive activation of the protein and tumour development. About 30% of all human tumours contain a mutation in a RAS allele, making this one of the most widely mutated human protooncogenes (Bos 1989, McCormick & Wittinghofer 1996).

Oncogenic mutations of H-RAS, K-RAS and N-RAS were among the first genetic changes to be

---

**Figure 1** Thyroid cancer genes (black) and the pathways they control. Mutually exclusive mutations of either RET/PTC, RAS or BRAF provide compelling genetic evidence for the critical role of the MAPK pathway. Additionally, recent studies point to an important role of the PI3K/AKT pathway in thyroid tumorigenesis. Adapted from Fagin 2004.
identified in tumours originating from the thyroid follicular epithelium, and numerous reports have documented their occurrence in many different types of thyroid tumours. Mutations of all three RAS genes are found in benign and malignant follicular neoplasms and in follicular variant PTC, and are believed to be one of the early steps in thyroid tumour formation (Lemoine et al. 1988, Namba et al. 1990). There are significant discrepancies related to the overall frequency of RAS mutations (ranging from 7 to 62%) and their prevalence in specific thyroid tumours (Wright et al. 1989, Suarez et al. 1990, Challeton et al. 1995). No consistent relationship between tumour histotype or biological behaviour and a particular pattern of RAS activation can be inferred from a review of the literature. In the largest series so far analysed, RAS mutations were not very common in follicular adenomas or in well-differentiated carcinomas (follicular or papillary) ranging from 5 to 10%. However, RAS mutations are more prevalent in poorly differentiated (55%) and anaplastic carcinoma (52%; Garcia-Rostan et al. 2003). Moreover, this group found a significant association between RAS mutations and poor survival, proposing RAS mutations as a marker for aggressive thyroid cancer behaviour.

As mutations in RAS are thought to be among the initiating molecular events in thyroid tumorigenesis, an inducible expression system in rat thyroid cells represents an excellent model to investigate the early biological events that take place after aberrant activation of RAS. Remarkably, conditional expression of H-RASV12 in rat thyroid cells induces strong apoptosis. This is surprising, because RAS inhibits apoptosis as part of its role in promoting expansion of the neoplastic clone in epithelial (Rak et al. 1995, Khwaja et al. 1997) and myeloid cells (Kinoshita et al. 1997), whereas in T lymphocytes and fibroblasts constitutive RAS activity can induce cell death (Chen et al. 1998). However, an important consideration should be taken into account. In the presence of TSH, H-RASV12 induces a transient increase in cell proliferation but later inhibits TSH-mediated cell growth via initiation of programmed cell death. These effects are mediated via the ERK and JNK signal transduction pathways and are only observed with concomitant stimulation of the cAMP signalling cascade (Shirokawa et al. 2000). By contrast, in the absence of TSH, acute expression of H-RASV12 does inhibit apoptosis and, thus, accelerates cell proliferation (TSH-independent growth). This means that the fate of thyrocytes within the first cell cycles after expression of oncogenic RAS is dependent on TSH levels. Those cells that loose TSH responsiveness and/or inactivate the apoptotic cascade through secondary events will undergo clonal expansion. RAS activation also displays evidence of DNA damage, manifesting as chromosome misalignment in mitosis, micronuclei formation and centrosome amplification (Saavedra et al. 2000). Interestingly, the same authors also demonstrated that DNA damage is not the only one responsible for activating apoptosis after acute RAS expression. Finally, RAS also induces dedifferentiation in a dose-dependent manner, and this is an early phenomenon. Although TSH-independent growth appears to be induced in the presence of both low and high levels of oncogenic RAS expression, only high levels of RAS expression are able to inhibit the activity of thyroid-specific genes, including TTF1 and PAX8, two transcription factors essential for maintenance of the thyroid differentiated state (De Vita et al. 2005). Additionally, the RAF/MEK/ERK cascade may act in concert with an as yet uncharacterised signalling pathway to repress TTF1 function and ultimately to inhibit thyroid cell differentiation.

Studies in vivo with transgenic mice have also given valuable information. A transgenic mouse line in which a human N-RAS (Gln61Lys) oncogene was expressed in thyroid follicular cells under control of the Tg promoter (Tg-N-RAS) was developed by Santoro’s group. Significantly, Tg-N-RAS mice developed thyroid follicular neoplasms; 11% developed follicular adenomas and ~40% developed invasive follicular carcinomas, in some cases with a mixed papillary/follicular morphology. About 25% of the Tg-N-RAS carcinomas displayed large, poorly differentiated areas, featuring vascular invasion and forming distant metastases in lung, bone or liver (Vitagliano et al. 2006).

In conclusion, RAS mutations are not restricted to a specific thyroid tumour type, and are present in follicular adenoma, follicular carcinoma, follicular variant of PTC and at a high frequency in poorly differentiated and anaplastic carcinomas. RAS mediates TSH-independent growth, TSH-dependent apoptosis, dedifferentiation and DNA damage leading to genomic instability, mainly through the RAF/MEK/ERK pathway. Other pathways may contribute to apoptosis, like the SAPK/JUNK pathway, and other unknown pathways in RAS-induced dedifferentiation (Table 1).

**RET/PTC rearrangements**

The RET gene encodes a transmembrane tyrosine kinase (TK) receptor whose expression and function is normally restricted to a subset of cells derived from the neural crest. In thyroid follicular cells, RET activation occurs through chromosomal recombination resulting
in expression of a fusion protein consisting of the intracellular TK domain of RET coupled to the N-terminal fragment of a heterologous gene. Several forms have been identified that differ according to the 5′ partner gene involved in the rearrangement. RET/PTC1 is formed by a paracentric inversion of the long arm of chromosome 10, leading to fusion of RET with a gene named H4/D10S170 (Grieco et al. 1990). RET/PTC2 is formed by a reciprocal translocation between chromosomes 10 and 17, resulting in the juxtaposition of the TK domain of c-RET with a portion of the regulatory subunit of RiaCAMP-dependent protein kinase A (Bongarzone et al. 1993). RET/PTC3 is also a result of an intrachromosomal rearrangement and is formed by fusion with the RFG/ELE1 gene (Santoro et al. 1994). Recently, several additional variants of RET/PTC have been observed in papillary carcinomas arising in children exposed to radiation after Chernobyl (Klugbauer et al. 1996, 1998). The fusion proteins dimerise in a ligand-independent manner due to motifs present in the N-terminal domains. This results in constitutive activation of the TK function of RET, autophosphorylation at selected tyrosine residues and initiation of intracellular signalling by engagement with effectors through specific tyrosine phosphorylated domains of the receptor. Three sites of tyrosine phosphorylation have been shown to function as docking sites for signalling molecules (Fig. 1): pY905 mediates the recruitment of the SH2 domain-containing proteins Grb7 and Grb10; pY1015 mediates the association between phospholipase Cγ (PLC) and RET (Borrello et al. 1996) and She and Frs2 interact with pY1062 mediating RAS/RAF/MAPK activation (Arighi et al. 1997, Melillo et al. 2001).

RET/PTC rearrangements were initially associated with PTC (Santoro et al. 1992), radiation exposure (Fugazzola et al. 1995, Klugbauer et al. 1995, Bounacer et al. 1997, Nikiforov et al. 1997) and young age (Ito et al. 1994, Bongarzone et al. 1996, Kumagai et al. 2004, Lima et al. 2004, Penko et al. 2005, Powell et al. 2005). However, RET mutations are not restricted to a malignant phenotype as they have also been found in sporadic follicular adenomas (Ishizaka et al. 1991), benign thyroid nodules (Bounacer et al. 1997, Elisei et al. 2001) and Hashimoto’s thyroiditis (Nikiforova et al. 2002a). Moreover, RET/PTCs are also frequently present in non-radiated thyroid tumours and in adults (Williams et al. 1996, Motomura et al. 1998, Smida et al. 1999, Elisei et al. 2001). Unknown ionising radiation exposure may explain these findings and/or other factors may act independently or in cooperation with radiation to trigger DNA damage leading to protooncogene activation. Reported frequencies of RET/PTC rearrangements in sporadic PTC vary widely among different countries, ranging from as low as 2.5% in Saudi Arabia to 59% in the United Kingdom. Several reasons have been advocated for such broad variability: ethnicity or genetic background in the occurrence of RET rearrangements (See review by Nikiforov 2002), the use of different detection methods, the genetic heterogeneity of the tumours (Zhu et al. 2006) and finally, we cannot rule out unknown environmental exposure to ionising radiations or to other mutagenic factors. Two different groups have reported a high frequency of RET/PTC rearrangements in papillary microcarcinomas: 42.3% in an Italian series (Viglietto et al. 1995) and 77% in a Canadian series (Sugg et al. 1998). Finally, there is some evidence that different types of RET/PTC rearrangements in papillary microcarcinomas: 42.3% in an Italian series (Viglietto et al. 1995) and 77% in a Canadian series (Sugg et al. 1998). Finally, there is some evidence that different types of RET/PTC rearrangements may be associated with distinct subtypes of PTC. RET/PTC1 tends to be more common in tumours with typical papillary growth and microcarcinomas and to have a more benign clinical course, whereas in some populations RET/PTC3 shows a strong correlation with the solid variant of papillary carcinoma (Nikiforov 2002). However, the presence of the mutation does not seem to influence the biological
behaviour of the tumour or its response to conventional treatment modalities (Pacini et al. 2000).

Like RAS mutations, RET/PTC rearrangements are thought to be tumour-initiating events. The early biological consequences of RET/PTC activation, as observed using an inducible system in rat thyroid cells, are an induction of apoptosis by both RET/PTC1 and RET/PTC3. However, in contrast to RAS mutations, none of the RET/PTCs bestowed cells with the ability to grow in the absence of TSH and no genomic instability was observed (Wang et al. 2003). The same group also demonstrated that the Y1062 phosphorylation site mediates RET/PTC-induced thyroid cell dedifferentiation and DNA synthesis through Shc, which activates the RAS/RAF/MEK/MAPK pathway (Knauf et al. 2003). Additionally, acute expression of the oncoprotein decreased TSH-mediated growth stimulation due to interference with TSH signalling by RET/PTC at multiple levels. Taken together, these data indicate that RET/PTC is a weak tumour-initiating event and that TSH action is disrupted by this oncoprotein at several points, and also predict that secondary genetic or epigenetic changes are required for clonal expansion. Finally, exposure of cell lines to ionising radiation results in induction of expression of RET/PTC within hours (Ito et al. 1993), supporting a direct role for radiation in the recombination of RET.

Thyroid-specific overexpression of either RET/PTC1 (Jhiang et al. 1996, Santoro et al. 1996) or RET/PTC3 (Powell et al. 1998) in transgenic mice leads to development of tumours with histological features consistent with PTC. RET/PTC1-transgenic mice develop thyroid tumours displaying thyroid hyperplasia and the main histological features of PTC. These tumours are slowly progressive and do not cause metastasis (Santoro et al. 1996). Transgenic mice expressing human RET/PTC3 develop thyroid hyperplasia and solid tumour variants of PTC that metastasise to regional lymph nodes (Powell et al. 1998). Buckwalter et al. (2002) investigated the contribution of the three signalling pathways triggered by the specific phosphorylated tyrosine domains of the receptor by characterising transgenic mice expressing thyroid-targeted RET/PTC1 mutants with phenylalanine substitutions at either Y905, Y1015 or Y1062. Tumour formation was significantly decreased in all of the mutants, but, in particular, in RET/PTC1-Y905F double mutants. This points to significant contributions by all of these pathways to RET/PTC-induced thyroid cell transformation. However, as tumours are still able to form in some transgenic mice from all three single mutant transgenic groups, the authors speculate that the signalling pathways involving any one of these three phosphorylation sites can be compensated by alternative redundant or complementary pathways and lead to thyroid tumour formation in vivo. This assumption has been confirmed partially in vitro in PCC13 cells, where neither Y1015 nor Y1062 alone are required for the RET/PTC-induced effects on growth and apoptosis. By contrast, in the same in vitro model there is an absolute requirement of Y1062 for RET/PTC-induced dedifferentiation, as determined by decreased expression of thyroid-specific gene products such as the sodium iodide symporter (NIS), Tg or PAX8. RET/PTC-mediated dedifferentiation requires activation of Shc/RAS/RAF/MAP kinase (Knauf et al. 2003).

Overall, RET mutations are present in sporadic PTC, in PTC arising after radiation exposure and in children. They are highly prevalent in microcarcinomas, have not been described in poorly differentiated and anaplastic carcinomas and are not related to poor prognosis. RET/PTC1 tends to be more common in tumours with typical papillary growth and microcarcinomas and tends to have a more benign clinical course, whereas RET/PTC3 shows a correlation with the solid variant of PTC and more aggressive tumour behaviour. Early biological events after RET/PTC activation are consistent with apoptosis and dedifferentiation, but RET/PTC is not able to induce TSH-independent growth or genomic instability, exerting a weak oncogenic drive. None of the RET/PTC tyrosine residues alone is absolutely required for tumour formation, and all appear to contribute to the ultimate phenotype to some extent.

**BRAF**

There are three isoforms of the serine–threonine kinase RAF in mammalian cells: ARAF, BRAF, and CRAF or RAFl. CRAF is expressed ubiquitously, whereas BRAF is expressed at higher levels in hematopoietic cells, neurons and testes (Daum et al. 1994). BRAF has a higher affinity for MEK1 and MEK2 and is more efficient in phosphorylating MEKs than other RAF isoforms (Peyssonnaux & Eychene 2001). The majority of the oncogenic mutations of BRAF destabilise the inactive BRAF structure, thereby promoting an active conformation and leading to a constitutive catalytic activation (Wan et al. 2004, Moretti et al. 2006). The V600E mutant of BRAF is one of the most prevalent somatic genetic events in human cancer and possesses the hallmarks of a conventional oncogene (Davies et al. 2002). The kinase activity of this mutant protein is greatly elevated; it constitutively stimulates ERK activity...
in vivo independent of RAS and potently transforms NIH3T3 cells.

The BRAFV600E mutation is the most common genetic change in PTC, present in about 29–83% of cases (see review by Xing 2005). Unlike RAS or RET/PTC rearrangements, BRAF mutations are unique to PTC and are not found in any other form of well-differentiated follicular neoplasm arising from the same cell type. BRAF mutations can occur early in the development of PTC, based on evidence that they are present in microscopic PTC (Nikiforova et al. 2003b). The tall cell (TC) variant papillary thyroid cancers, widely regarded as more aggressive, have a particularly high prevalence of BRAF mutations (Nikiforova et al. 2003b). Undifferentiated or anaplastic carcinomas arising from pre-existing papillary thyroid cancers also have a significant prevalence of BRAF mutations, whereas those arising from pre-existing follicular carcinoma do not (Namba et al. 2003, Nikiforova et al. 2003b, Begum et al. 2004, Soares et al. 2004, Xing et al. 2004b, Quiros et al. 2005). Some studies, but not all, have found BRAF to be associated with aggressive clinicopathological features such as advanced clinical stages and extrathyroidal extension (Namba et al. 2003, Nikiforova et al. 2003b, Xing et al. 2005, Riesco-Eizaguirre et al. 2006). Moreover, we have demonstrated, along with another group, that BRAFV600E is associated with a high recurrence rate during the early follow-up of the patients and that the majority of these recurrences have no avidity for radioiodine (131I) and, thus, are unresponsive to 131I treatment, and point out that this genetic event is a new biological marker that predicts poor prognosis and resistance to treatment of thyroid cancer (Xing et al. 2005, Riesco-Eizaguirre et al. 2006). We further confirmed that these data in studies in vitro in which assessment of NIS expression by immunohistochemistry in human tumour samples and transfection experiments in rat thyroid cells demonstrated that BRAFV600E sharply impairs both NIS expression and NIS trafficking to the membrane.

Like RAS mutations and RET/PTC rearrangements, BRAF mutations are thought to be a tumour-initiating event. The early biological consequences of BRAF activation show that BRAF induces apoptosis and does not confer on cells the ability to grow in the absence of TSH, because of concomitant stimulation of both DNA synthesis and apoptosis, resulting in no net growth in the cell population (Mitsutake et al. 2005). BRAF-induced apoptosis was also seen in the presence of TSH. Acute BRAFV600E expression in PCCL3 cells induces dedifferentiation and genomic instability (Mitsutake et al. 2005). These data indicate that BRAFV600E expression confers little growth advantage on thyroid cells because of concomitant activation of DNA synthesis and apoptosis. However, in contrast to RET/PTC, and similarly to RAS, BRAFV600E may facilitate the acquisition of secondary genetic events (or primary epigenetic events) through induction of genomic instability, which may account for its aggressive properties (Mitsutake et al. 2005). Both RET/PTC and BRAF decrease expression of the TSH receptor (TSHR). However, the mechanisms by which RET/PTC and BRAF interfere with TSH action distal to the receptor differ in important aspects. In contrast to RET/PTC, BRAF activation does not impair key activation steps distal to the TSHR, such as forskolin-induced adenyl cyclase activity or cyclic AMP-induced DNA synthesis (Mitsutake et al. 2005).

Thyroid-specific overexpression of the mutant BRAF in transgenic mice leads to development of tumours with histological features consistent with invasive PTC, which exhibit foci of classic features, foci of TC features and foci of poorly differentiated carcinomas. These mice had a 30% decrease in survival at 5 months (Knauf et al. 2005). This closely recapitulates the phenotype of BRAF-positive PTCs in humans. These data indicate that BRAFV600E is present exclusively in PTC, particularly in the classic and TC variant, and in poorly and anaplastic carcinomas that most likely arise from PTC. It may be an alternative tumour-initiating event in PTC, and tumours with this genotype may carry a less favourable prognosis.

**Overview**

There are two kinds of evidence demonstrating that the genetic events mentioned above are tumour-initiating events. One of them is that these mutations are present in microcarcinomas. The other one arises from studies on transgenic mice, where the specific activation of these oncogenes in the thyroid provokes a tumour phenotype very similar to the one seen in humans. Assuming that these genetic events are tumour-initiating events, the study of the early biological events using inducible systems represents an excellent model to provide new insights into thyroid tumorigenesis. Oncogene-induced apoptosis is an early and common biological event present in the three oncogenic phenotypes. This phenomenon usually does not happen in other epithelial cells, where the oncogene itself triggers mechanisms that are able to override apoptosis. Dedifferentiation is also widely seen after early oncogene induction, although the mechanisms and pathways involved differ among them. TSH-independent growth is seen only after RAS induction. This trait is only achieved by BRAF and
RET after stably transflecting the cell, indicating that TSH-independent growth occurs in a second step during the tumorigenesis in BRAF- and RET-positive tumours. Finally, BRAF and RAS induce genomic instability, while RET does not. Whether this genomic instability induces secondary genetic events that confer more aggressiveness to both tumour phenotypes is something that needs to be elucidated. It should be noted that other signalling pathways and molecular targets, although not directly activated through genetic mutations, may prove to be crucial for thyroid cancer progression and thus appropriate for targeted inhibition (Table 1).

**The phosphatidylinositol 3-kinase (PI3K)/Akt pathway**

Activation of Akt plays a pivotal role in fundamental cellular functions such as cell proliferation and survival by regulating the function of many cellular proteins. It has been reported that alterations to the PI3K/Akt signalling pathway are frequent in human cancer. Constitutive activation of the PI3K/Akt pathway occurs due to mutations or amplification of the *PIK3CA* gene encoding PI3K or the Akt gene, or as a result of inactivating mutations in components of the pathway, for example PTEN (phosphatase and tensin homologue deleted on chromosome 10), which inhibit the activation of Akt.

The first evidence of the implication of this pathway in thyroid tumours was provided by a syndrome called Cowden disease. Germline mutations of the PTEN gene confer predisposition to Cowden disease, a condition characterised by development of hamartomas in multiple organs, benign thyroid disorders such as multinodular goitre and adenoma, and increased risk of thyroid (mostly of the follicular type), breast and other cancers (Eng 1998). However, in sporadic thyroid neoplasm, a role for PTEN has not been firmly established. Loss of heterozygosity (LOH) was found in 27% of follicular carcinomas and 7% of follicular adenomas, one of which was a small hemizygous deletion (Halachmi et al. 1998), and loss or reduction of PTEN expression as well as inappropriate subcellular compartmentalisation has been reported in thyroid tumours (Bruni et al. 2000, Gimm et al. 2000, Vasko et al. 2004). The absence of somatic PTEN mutations has lead to the screening for other genes involved in the PI3K/Akt pathway. The potential relevance of this pathway is also supported by the observation that Akt expression and activation is increased in thyroid cancers, particularly in FTC (Ringel et al. 2001, Vasko et al. 2004). Additionally, PTEN loss in transgenic mice and subsequent activation of the PI3K/Akt pathway causes goitre and follicular adenomas but is not sufficient for malignant transformation of thyroid cells (Yeager et al. 2007). Recently, Garcia-Rostan et al. (2005) reported in a European series that somatic mutations within the PI3K catalytic subunit, *PIK3CA*, is present in 23% of anaplastic carcinomas and in 8% of FTC and is likely to function as an oncogene in anaplastic thyroid cancer (ATC) and less frequently in well-differentiated thyroid carcinomas. The authors also argued for a role of *PIK3CA* targeting in the treatment of ATC patients. This *PIK3CA* mutant has also been observed in colorectal, gastric, breast and ovarian cancers, and in high-grade brain tumours (25–40%). However, Wu et al. (2005) did not find any *PIK3CA* gene mutations in an American series of thyroid tumours. Instead, they observed *PIK3CA* gene amplification in 4 of 34 (12%) benign thyroid adenomas, 3 of 59 (5%) PTC, 5 of 21 (24%) FTC, none of 14 (0%) medullary thyroid cancers and in 5 of 7 (71%) thyroid tumour cell lines.

In an attempt to investigate the overall occurrence of genetic alterations in the PI3K/Akt pathway in sporadic thyroid cancer, Hou et al. (2007) investigated the presence of *PIK3CA* copy number gain and mutation, RAS mutation and PTEN mutation in a large series of thyroid tumours. However, RET/PTC rearrangements, which also activate the PI3K pathway, were not included. The occurrence of any of these genetic alterations was found in 25 of 81 (31%) benign thyroid adenomas, 47 of 86 (55%) follicular thyroid cancers, 21 of 86 (24%) papillary thyroid cancers and 29 of 50 (58%) ATCs, with FTC and ATC most frequently harbouring these genetic alterations. *PIK3CA* copy gain was associated with increased *PIK3CA* protein expression. Although Hou et al. describe a mutual exclusivity of these alterations in well-differentiated carcinomas and adenomas, they did not observe this in anaplastic carcinomas, arguing for a role in the progression of FTC to ATC as the genetic alterations of this pathway accumulate.

More studies in *vitro* and in *vivo* are needed to elucidate the role this aberrant activation of PI3K/Akt pathway plays in thyroid tumorigenesis, particularly in FTC and ATC. Several small molecules designed to specifically target PI3K/Akt have been developed and induced cell cycle arrest or apoptosis in human cancer cells in *vitro* and in *vivo*. Moreover, the combination of an inhibitor with various cytotoxic agents enhances the anti-tumour efficacy (Osaki et al. 2004). Therefore, specific inhibition of the activation of Akt may be a valid approach to treating thyroid malignancies, particularly FTC and ATC.
The p53 pathway

Inactivating point mutations of the p53 tumour suppressor gene are highly prevalent in anaplastic and poorly differentiated thyroid tumours, but not in well-differentiated papillary or follicular carcinoma (Ito et al. 1992, Fagin et al. 1993). These data imply p53 inactivation as an important step in late stage progression of thyroid cancer. Thyroid cells carrying a mutated p53 gene did not form colonies in soft agar or tumours in athymic mice, suggesting that a mutation of the p53 gene is not sufficient for the induction of the malignant phenotype, and probably cooperation with other oncogenes is necessary to accomplish full malignancy. However, a mutated p53 gene results in a marked loss of the differentiated phenotype in the rat thyroid cell line PCCl3, including inhibition of the expression of the thyroid-specific transcription factor PAX8 (Battista et al. 1995). Conversely, re-expression of wt-p53 activity in undifferentiated thyroid carcinoma cell lines inhibits cell proliferation and restores differentiation (Fagin et al. 1996, Moretti et al. 1997).

The PAX8/PPARγ rearrangement

This translocation fuses the thyroid-specific transcription factor PAX8 gene with the PPARγ gene, a ubiquitously expressed transcription factor that has been shown to play an important role in regulating genes involved in adipocyte differentiation and lipid metabolism. It involves a chromosome 3p25 and 2q13 translocation (Kroll et al. 2000) creating a fusion gene, encompassing the promoter and proximal 50 coding sequence of the thyroid-specific transcription factor PAX8 gene and most of the coding sequence of the PPARγ gene. PAX8/PPARγ rearrangement has been identified in a significant proportion of FTC (36–45%), follicular adenoma (FA; 4–33%), follicular variant of PTC (37.5%) or Hürthle cell carcinoma (Kroll et al. 2000, Marques et al. 2002, Nikiforova et al. 2002b, 2003a, French et al. 2003, Castro et al. 2006).

The mechanism of transformation induced by PAX8/PPARγ is still unclear. It has been shown to have a dominant negative effect on thiazolidinedione-induced transactivation by PPARγ (Kroll et al. 2000) and overexpression of wild-type PPARγ1 in thyroid cancer cell lines inhibits cell growth, an effect that is further enhanced by PPAR agonist (Martelli et al. 2002). However, another group did not find this dominant negative effect in the rat thyroid cells FRTL5 nor in immortalised human thyroid cells, raising the question whether the transforming properties of PAX8/PPARγ can be attributed only to inhibition of PPARγ function (Au et al. 2006).

Epigenetics of thyroid cancer

Epigenetic modifications, such as histone deacetylation and DNA methylation, play an important role in the regulation of gene expression and are likely to be involved in carcinogenesis. DNA methylation is a covalent modification of cytosine residues that occurs at the dinucleotide sequence CpG in vertebrates. Nearly half of all human genes have CpG islands associated with transcriptional start sites. Unmethylated CpG islands are seen in highly transcribed genes, whereas heavily methylated CpG islands result in heritable inhibition of gene transcription (Baylin 1997). Although overall DNA methylation is often decreased in cancers, CpG islands in critical gene promoter regions can become hypermethylated, resulting in heritable inhibition of gene expression. Abnormal patterns of DNA methylation are observed consistently in human tumours, including benign and malignant human thyroid tumours (Matsuo et al. 1993), usually occurring as an early event (Counts & Goodman 1995).

Aberrant methylation affecting thyroid-specific genes in benign and malignant thyroid tumours have been described by several groups, demonstrating a role for this mechanism in thyroid dedifferentiation. Affected genes include the TSHR gene (Xing et al. 2003b), the Pendred syndrome gene SLC26A4 (Xing et al. 2003a) and the NIS gene (Venkataraman et al. 1999, Neumann et al. 2004). The observation of epigenetic alterations of the NIS promoter has opened new approaches to recovering NIS expression. Demethylating agents and inhibitors of histone deacetylase have been used successfully in vitro to restore iodine uptake in thyroid cancer cell lines (see review Riesco-Eizaguirre & Santisteban 2006).

Recently, several tumour suppressor genes and candidate tumour suppressor genes have been shown to be silenced due to aberrant methylation in thyroid cancer. Examples of these genes include RASSF1A (Schagdarsurengin et al. 2002, Xing et al. 2004a), genes encoding the cyclin-dependent kinase inhibitors p15INK4b and p16INK4a (Elisei et al. 1998, Boltze et al. 2003), the tissue inhibitor of metalloproteinase-3 (TIMP3; Hu et al. 2006), SLC5A8 (also called sodium monocarboxylate transporter (SMCT; Hu et al. 2006), death-associated protein kinase (DAPK; Li et al. 2003), retinoic acid receptor β-2 (RARβ-2; Hoque et al. 2005) and PTEN (Alvarez-Nunez et al. 2006). Methylation of some of these genes (TIMP3, SLC5A8 and DAPK) was significantly associated with several aggressive features of PTC, and may have a role as potential prognostic markers (Hu et al. 2006). However, there is still a lot to be
learned about all these silencing events and their potential pathogenic significance is still uncertain. For instance, although restoration of RASSF1A expression inhibits tumorigenicity in vitro and in vivo in lung cancer, the biological function of RASSF1 and its isoforms (A, B, C, D, E, F and G) is still under investigation. Interestingly, mutational inactivation of this gene is very rare (2%), and the main mechanism of its inactivation is through promoter methylation and LOH. Future studies focussing on the mechanisms underlying these epigenetic alterations need to be done in order to have a better view of the pathogenic significance in thyroid tumorigenesis.

The genomics of thyroid cancer

Tumour gene expression profiling by DNA microarrays has brought new important clues to our understanding of cancer pathophysiology and simultaneously has provided clinically valuable information on many malignant neoplasms. Taking advantage of this new approach, several studies have given hints about the molecular pathways involved in thyroid tumorigenesis and may provide new biomarkers for clinical use. Gene expression studies are beginning to be applied to thyroid cancer (Huang et al. 2001, Barden et al. 2003, Aldred et al. 2004, Chevillard et al. 2004, Finley et al. 2004, Frattini et al. 2004, Mazzanti et al. 2004, Wreesmann et al. 2004, Giordano et al. 2005, Jarzab et al. 2005) and should yield significant improvements in thyroid cancer diagnosis, prognosis and treatment.

In the first study performed using this methodology (Huang et al. 2001), results from eight PTC tumours were compared with normal thyroid tissue from the same eight individuals. Although PTC is clinically heterogeneous, the authors concluded that PTC is characterised by consistent and specific global expression patterns. They detected differential expression of genes that were previously known to be altered in PTC, validating the feasibility of their experimental approach, such as MET, LGALS3 (galectin 3), KRT19, DPP4, MDK, TIMP1 and FN1 (fibronectin).

They also reported numerous additional genes to be differentially expressed, proposing some of them as candidate clinical markers, since they were significantly upregulated in the great majority of more than 40 PTCs. Finally, these findings gave clues about the molecular pathways involved in PTC. Genes related with molecular adhesion were clearly overexpressed in PTC versus normal thyroid, whereas genes underexpressed in PTC included tumour suppressors, thyroid function-related proteins and fatty acid binding proteins. In a similar study, Wasenius et al. confirmed in a larger series much of the data seen by Huang et al. but focused on different candidate genes that could serve as biological markers for PTC.

Using more advanced bioinformatics methods that were not used before addressing thyroid cancer by other groups, Jarzab et al. (2005) demonstrated that the difference between tumour and normal samples was the major source of variability in the gene expression pattern of thyroid tissues, confirming the results obtained by Huang et al. (2001) However, despite very distinct changes in expression, none of the genes proved to be an ideal single marker of PTC in an independent set of PTCs analysed by Q-PCR. This study confirmed the overexpression of cell adhesion genes that was indicated by the aforementioned microarray study. This group of genes, possibly related to invasion and metastasis processes, constituted the most numerous gene ontology class. Concerning signal transduction-related genes, the very distinct upregulation of MET is a consistent feature also found in other genomic studies of PTC (Wasenius et al. 2003, Finley et al. 2004). The authors also emphasised that among the five transcription factors upregulated, RXR-γ was a novel gene, which could have implications for the response to retinoids in thyroid cancer. However, genomic profiling provides a very partial view of functionality and a wide spectrum of descriptive data that it is not easy to interpret. Also, one of the major drawbacks of this kind of approach is its lack of reproducibility.

Another study made a comparative analysis of microarray expression data for PTC and FTC, the two most common forms of non-medullary thyroid carcinoma. Although there were some similarities between the two tumour types among underexpressed genes, distinct expression patterns could be observed. Five genes (CITED1, CAV1, CAV2, IGFBP6 and CLDN10) were identified that collectively distinguish PTCs from FTCs (Aldred et al. 2004). Although PTC is characterised by consistent and specific global gene expression patterns, in a very interesting study, Giordano et al. (2005) observed a relationship between morphologic subtype of PTC and gene expression. The follicular variants (FV) were tightly grouped in the PCA plot, and the TC variants were loosely grouped among the remaining classic types (CT). Interestingly, the authors further observed that incorporating the BRAF, RET/PTC and RAS mutational status revealed a strong relationship between mutation and gene expression, with PTCs closely grouped for each mutation type. Interestingly, tumours with BRAF mutations displayed either TC or classical variant morphology; tumours with RET/PTC rearrangements displayed predominantly the classical morphology and
tumours with RAS mutations exclusively displayed the follicular variant morphology. Tumours with no apparent mutation predominantly had the follicular variant morphology. Also, the analysis revealed that mutation was more strongly correlated with gene expression than with morphology. Frattini et al. (2004) performed a similar though smaller profiling study and, although they showed no strong differences in global gene expression in PTC, they found some expression differences between tumours with RET or NTRK1 rearrangements and tumours with BRAF mutations.

The striking relationship between gene expression and genotype indicates that mutations affecting the RET/RAS/BRAF/MAPK pathway are the predominant source of gene expression variation and suggests that they represent the earliest mutational events occurring in PTC, resulting in persistent and distinct patterns of abnormal gene expression. In addition, the absence of premalignant lesions in well-differentiated PTC, in contrast to other epithelial tumour types such as colon carcinoma, suggests that PTCs are the morphological manifestation of single dominant activating mutations. This finding suggests that these mutations are able to signal through alternative pathways and may confer discrete mutation-specific phenotypical and biological features on tumours. For instance, the authors observed that among the mutation-specific differentially expressed genes in PTC, some have roles in signal transduction such as VAV3, a member of the VAV oncogene family involved in PI3K signalling and subsequent AKT activation. Its preferential expression in PTCs with RET/PTC and RAS mutations may provide evidence that these PTCs signal more through PI3K than MAPK pathways. The identification of TM7SF4 as one of the most preferentially expressed genes in PTCs with BRAF mutations has implications for the immunological aspects of PTC. TM7SF4 is a transmembrane protein expressed in dendritic cells and has a role in antigen processing and initiation of the immune response. Its expression profile lead to the authors to speculate that BRAF mutation has a unique role in initiating an immune response in PTC.

As mentioned before, genomic profiling has its limitations and provides a very partial view of functionality. For instance, despite the variability in the genes mentioned above, the expression of genes related to DNA replication, cell cycle, mRNA splicing and protein biosynthesis showed much less variation than could be expected (Jarzab et al. 2005). We should also bear in mind that the functions of selected genes may be related to the stromal component of the tumour. Thyroid tumours consist of neoplastic cells intermingled irregularly with normal (connective tissue and vessels) and reactive (stromal and immune) cells (Kroll 2002). Quantitative relations between these components may vary between patients and even inside one tumour. Most microarray studies include tumour fragments containing more than 80–90% of tumour cells and some authors recommend investigation of microdissected cells (Jarzab et al. 2005).

Apart from all this genomic profiles made in human tumour samples, several in vitro studies have tried to define the functional role of selected genes in tumoral transformation, particularly in metastasis progression. In thyroid cell lines, the activation of unique patterns of gene expression exerted by the three main oncogenes have also been demonstrated. Melillo et al. (2005) examined the transcriptional profile of rat thyroid PCCL3 cells stably expressing RET/PTC, RAS or BRAF. A large fraction of the genes activated by RET/PTC3 were also activated by RAS or BRAF, consistent with the primary role of the RET/RAS/BRAF/MAPK pathway in transformation. This was confirmed for a subset of genes by use of RNA interference (RNAi) for BRAF or by MEK inhibitors. However, there were relatively large sets of genes specifically modulated by each oncogene. Interestingly, they also showed a critical role of this pathway in stimulating cell motility, mediated in part by induction of the chemokines CXCL1 and CXCL10 (Melillo et al. 2005). In a slightly different model, Mesa et al. (2006) examined the pattern of gene expression after conditional activation of these oncoproteins in thyroid PCCL3 using an inducible system. Metalloproteinases were preferentially induced by BRAF, particularly matrix metalloproteinase 3 (MMP3), MMP9 and MMP13. Enhanced production of MMPs in the tumour environment, either by stromal or by tumour cells, is believed to be an important determinant of tumour invasion. As BRAF was associated with markedly increased invasion into Matrigel compared with cells expressing RET/PTC3, the preferential induction of MMPs by BRAF could explain in part the more invasive behaviour of thyroid cancers with BRAF mutations (Mesa et al. 2006). Furthermore, BRAF-induced expression of matrix metalloproteinase and cell invasion into matrigel seems to be mediated NF-κB pathway (Palona et al. 2006), although the intrinsic molecular mechanism of BRAF-induced metastatic progression remains to be elucidated.

**An integrated perspective of thyroid cancer pathogenesis**

We now have compelling evidence that indicate that PTC requires constitutive activation of the
RTK/RAS/RAF/MAPK pathway for tumour initiation and progression. Similarly, we are starting to have more evidence that the PI3K/AKT pathway seems to play an important role in tumour initiation and progression of FTC. The p53 pathway appears to be involved exclusively in tumour progression of both phenotypes, as they are only present in ATC. One can speculate that tumour phenotype may differ in part according to the pre-eminence of the pathway activated and its occurrence early or later in tumour progression. This point of view is probably too simplistic but it is a good starting point that, for instance, has lead to the development of new targeted therapies aimed at blocking these pathways. In addition to the RTK/MAPK, PI3K/AKT and p53 pathways, there are others that have a role in many tumour types, including those involving the adenomatous polyposis coil (APC), glioma-associated oncogene (GLI), hypoxia-inducible transcription factor (HIF)-1 and SMADs. However, their role in thyroid cancer is yet to be discovered.

The nature of the tumour-initiating event is also important in determining the tumour phenotype. PTCs harbouring RET/PTC variants, RAS and BRAF mutations exert its own oncogenic drive, exhibiting distinct pathological features, genomic profiles, epigenetic changes and biological behaviour. Follicular variant of PTC commonly has RAS mutations, the classical and TC variant PTC have BRAF mutations and solid variants of PTC harbour RET/PTC3 rearrangements. Obviously, there has been an attempt to study the clinical significance of all these genetic events. Unfortunately, as RAS or PAX8/PPARγ is not restricted to a malignant phenotype, they are not useful as biological markers to discriminate between follicular adenoma and carcinoma. On the contrary, BRAF mutations are common and specific for PTC. FNAC is routinely used in the preoperative diagnosis of thyroid nodules and about 15–20% of FNAC are undeterminate. A BRAF mutation can be reliably detected in cells aspirated from a FNAC of a thyroid nodule and it has been shown to establish the diagnosis of PTC in 16% of carcinomas within the indeterminate group (Cohen et al. 2004, Xing et al. 2004e). Besides, the prognostic impact of BRAF and its role in discriminating between low- and high-risk patients is still a controversial matter, pointing to the need of prospective randomised studies in large series of patients. The observation that BRAF-positive tumours have lower levels of NIS expression and are associated with recurrences that have lost the ability to concentrate radioiodine may pave the way to elucidate the mechanisms underlying the dedifferentiation process that affects some thyroid cancer metastasis. Indeed, new strategies are needed to treat the challenging situation of elevated Tg and negative radioiodine scans in patients with thyroid cancer.

Targeted therapies in thyroid cancer

Since thyroid follicular cancer conserves a certain degree of differentiation, one logical therapeutic approach is to redifferentiate the cells and reinude endogenous NIS expression so that radioiodine treatment can be performed. Before targeted therapy became feasible, many groups focused on this strategy and several compounds, also known to have tumour-inhibitory effects, have partially succeeded in reaching this goal. Among them, the most well known has been retinoic acid (RA). Several clinical trials have been done using RA in order to increase radioiodide uptake and improve clinical outcome of patients with recurrent thyroid cancer (see review by Dohan et al. 2003). In general terms, radioiodide uptake was improved in 20–42% of the cases, but tumour shrinkage was observed in very few cases after 131I treatment. The largest study was done on 50 iodide scan-negative patients: 26% had a significant increase in radioiodide uptake, but only 16% had reduced tumour volume (Simon et al. 2002). Other compounds such as Troglitazone, HDAC inhibitors and demethylating agents are currently being tested with promising results (see review by Riesco-Eizaguirre & Santisteban 2006).

The prevalence of activating BRAF mutations, RET/PTC rearrangements and RAS mutations and consistent downstream activation of ERK suggests that activation of the MAP kinase signalling cascade may be an obligatory step in the transformation of thyrocytes. Allegedly, such dependency may represent a point of therapeutic attack (Table 2). However, we should bear in mind that, as we have explained before, deregulation of other pathways and/or secondary genetic events may be playing important roles in thyroid tumorigenesis. Several lines of experimental evidence affirm the notion that targeting RAF activity can be a good starting point. First, depletion of RAF, by small interfering RNA, in BRAF-positive thyroid cancer cell lines inhibits both ERK activation and proliferation while abrogating tumour xenograft formation (Salvatore et al. 2006). Secondly and most interestingly, RAF activity seems to be required for the transforming effects of the non-overlapping mutations of the other oncopgenes signalling through the MAPK pathway. Indeed, RAF binds to and is a direct effector for RAS (Troppmair et al. 1994), and selective knockdown of BRAF (but not CRAF) abrogates RET/PTC3-induced ERK phosphorylation in thyroid cells (Melillo et al. 2005, Mitsutake et al. 2006). These data suggest
that blocking RAF activity may be a logical approach to interfere with the effects of RET/PTC, RAS and BRAF oncoproteins.

Given the prominence of this ‘oncogene addiction’ phenotype involving the MAP kinase pathway in thyroid and other cancer types, several small molecules that target this pathway are currently being tested \textit{in vitro} and \textit{in vivo}. Sorafenib (BAY 43-9006) was one of the first compounds to be evaluated in clinical trials. This biaryl urea exhibits \textit{in vitro} inhibition of both wild-type and mutant BRAF activities in the 20–40 nM range (Ahmad \& Eisen 2004). Sorafenib is a multi-kinase inhibitor that also inhibits CRAF and VEGFR3 at low concentrations, while inhibiting platelet-derived growth factor receptor (PDGFR), VEGFR-2, c-KIT and FLT3 at higher concentrations \textit{in vitro}. Despite this robust effect \textit{in vitro}, the potency of this compound against MAPK activity in whole cells has proved more variable. Salvatore \textit{et al.} (2006) reported half-maximal inhibitory concentrations (IC50) values for sorafenib against BRAF-positive thyroid cancer cell lines of 1 \textmu M to reduce proliferation, and 5 \textmu M to virtually arrest growth (50- to 100-fold higher than the required concentration for \textit{in vitro} kinase inhibition. In xenografts, daily administration of 60 mg/kg sorafenib attenuated tumour growth, almost completely inhibited phospho-MEK activity and reduced Ki67/MIB-1 immunolocalisation (a proliferation marker). It also diminished the number of blood vessels in the xenograft, demonstrating an antiangiogenic effect (Salvatore \textit{et al.} 2006). Carlomagno \textit{et al.} (2006) obtained similar results using sorafenib against oncogenic RET/PTC-positive thyroid cancer cell lines. In this case, the IC50 was significantly lower (50 nM) and the same oral doses in xenografts (60 mg/kg per day) reduced tumour growth significantly.

Despite its promising preclinical properties, the preliminary efficacy data for sorafenib in patients with thyroid cancer appear more modest, yet significant. In a recent phase I/II clinical trial, a total of 46 patients with iodine-refractory measurable metastatic thyroid carcinoma were given sorafenib at a dose of 400 mg/day for a median of 12 weeks (range 3–28 weeks). The group was heterogenous, including papillary, follicular, Hürthle and anaplastic carcinomas. Among the 28 evaluable patients, only 5 had progressive disease during the treatment, 12 (42.8\%) had serum Tg decreased significantly and 5 out of 11 patients (45\%) in whom MRI was performed showed an average decrease of 57\% on exchange rate that was concordant with Tg response (Kloos \textit{et al.} 2005).

Sorafenib received FDA approval for the treatment of metastatic renal cancer, a malignancy where BRAF mutations have not been observed. In this case, sorafenib is believed to have a prominent antiangiogenic effect, and therefore its clinical efficacy may derive more from its anti-VEGF activity than from BRAF blockade.

Other RAF inhibitors have been tested for thyroid cancer, yet they did not show better \textit{in vitro} potencies than sorafenib. For example, AAL881 and LBT613 (Novartis, Cambridge, MA, USA) show a pharmacologic IC50 of > 100 nM against wild-type and mutant BRAF \textit{in vitro}. In thyroid carcinoma cell lines, the IC50 rose up to 10 \textmu M (tenfold higher than sorafenib), and some toxicity was reported \textit{in vivo}, particularly with LBT613. Despite these pharmacokinetic concerns, both compounds were effective growth inhibitors of poorly differentiated thyroid cancer cell lines with either RET or RAF mutations (Ouyang \textit{et al.} 2006).

### Table 2: Targeted therapies in thyroid cancer

<table>
<thead>
<tr>
<th>Agent</th>
<th>Target</th>
<th>Additional targets</th>
<th>Structure</th>
<th>IC50 (nM)</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAY 43-9006(^a) (Sorafenib)</td>
<td>RAF</td>
<td>VEGFR, PDGFR</td>
<td>Biaryl urea</td>
<td>12</td>
<td>Clinical study phase I/II</td>
</tr>
<tr>
<td>AAL881(^b)</td>
<td>RAF</td>
<td>C-ABL, KDR</td>
<td>Isoquinoline</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>LBT613(^b)</td>
<td>RAF</td>
<td>RET, C-ABL, KDR</td>
<td>Isoquinoline</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>AMG 706</td>
<td>VEGFR</td>
<td>KIT, PDGFR</td>
<td>Carboxamide</td>
<td>10</td>
<td>Clinical study phase I/II</td>
</tr>
<tr>
<td>ZD6474(^c)</td>
<td>RET</td>
<td>VEGFR, EGFR</td>
<td>Quinazoline</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>PP1</td>
<td>RET</td>
<td>SRC</td>
<td>Pyrazolopyrimidine</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>PP2</td>
<td>RET</td>
<td>SRC</td>
<td>Pyrazolopyrimidine</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>CEP-701</td>
<td>RET</td>
<td>NTRK</td>
<td>Indolocarbazole</td>
<td>&lt;100</td>
<td>Medullary thyroid carcinoma</td>
</tr>
<tr>
<td>CEP-751</td>
<td>RET</td>
<td>NTRK</td>
<td>Indolocarbazole</td>
<td>&lt;100</td>
<td>Medullary thyroid carcinoma</td>
</tr>
<tr>
<td>Plitidespin(^d)</td>
<td>Unknown</td>
<td>–</td>
<td>Marine derivative</td>
<td>10</td>
<td>Anaplastic thyroid carcinoma</td>
</tr>
<tr>
<td>PS-341(^e) (Bortezomib)</td>
<td>NF-(\kappa)B</td>
<td>–</td>
<td>Boronic acid dipeptide</td>
<td>5–20</td>
<td>Anaplastic and medullary</td>
</tr>
</tbody>
</table>

\(^a\)Bayer/Onyx; \(^b\)Novartis; \(^c\)AstraZeneca; \(^d\)PharmaMar; \(^e\)Janssen-Cilag.
generation small molecule inhibitors of RAF and MEK that exhibit \textit{in vitro} potencies exceeding that of sorafenib are currently being investigated, i.e. in BRAF-mutant melanoma. In addition, a series of small molecules has been shown to selectively target the V600E mutant of BRAF, resulting in submicromolar IC50 values (http://www.plexxicon.com). Presumably, these and other emerging RAF inhibitors may provide a more robust effect against MAP kinase activity in clinical trials.

Another small-molecule multikinase inhibitor, AMG 706, is a new orally bioavailable ATP-competitive inhibitor of VEGFR1, VEGFR2 and VEGFR3. In addition, AMG 706 inhibits Kit and platelet-derived growth factor receptor (PDGFR), two receptor tyrosine kinases (RTKs) related to VEGFR and implicated in the pathogenesis of several human cancers. AMG 706 inhibits VEGF-induced cell proliferation and vascular permeability and induces tumour regression \textit{in vivo} by selectively targeting neovascularisation in tumour cells (Polverino \textit{et al.} 2006). From a phase 1 study performed on a large series of patients with solid tumours (Rosen ASCO 2005; Herbst EORTC 2005), encouraging antitumour activity was seen in a subset of patients with thyroid cancer. Seven patients with iodine-refractory metastatic thyroid cancer received this compound orally for a median time of 141 days. Histologies were PTC (3), FTC (1), Hürthle cell (1), anaplastic (1) and medullary thyroid carcinoma (MTC) (1). Three patients (one PTC, one FTC and one MTC) showed a partial response consistent with reduced tumour volume and a decrease of tumour markers. The authors concluded that AMG 706 appeared to be well tolerated and, although the series was very small, this inhibitor displayed a promising antitumour activity in multiple histological subtypes of thyroid cancer (Boughton \textit{et al.} 2006).

Quinazolines are also some of the most promising inhibitors of RTKs (Levitzki 1999). For instance, ZD1839 (Iressa) is a potent and selective inhibitor of the EGFR and is currently in advanced clinical development (Ciardiello \textit{et al.} 2001). Another anilino-quinazoline, ZD6474, has been shown to be a selective inhibitor of the VEGF receptor-2 (flk-1/KDR) tyrosine kinase (Wedge \textit{et al.} 2002). Interestingly, this last compound has also been shown to inhibit the enzymatic and transforming activity of RET oncoproteins and arrests the development of RET/PTC3-induced tumours in nude mice. This low molecular weight tyrosine kinase inhibitor blocks the enzymatic activity of RET-derived oncoproteins at a half maximal inhibitory concentration of 100 nM \textit{in vitro} and blocks \textit{in vivo} phosphorylation and signalling of the RET/PTC3 and RET/MEN2B oncoproteins. RET/PTC3-transformed cells lost proliferative autonomy and showed morphological reversion after treatment with ZD6474. This compound also prevented the growth of two human PTC cell lines that carry spontaneous RET/PTC1 rearrangements. The authors concluded that targeting RET oncopgenes with ZD6474 might offer a potential treatment strategy for carcinomas sustaining oncogenic activation of RET such as PTC and medullary thyroid carcinomas (Carломagno \textit{et al.} 2002).

The proteasome inhibitor bortezomib (PS-341, Velcade, Janssen-Cilag, Belgium) has been approved by the Food and Drug Administration for the treatment of multiple myeloma. The inhibition of inhibitory-κB degradation, which leads to inactivation of the transcription factor nuclear factor-κB (NF-κB), seems to be its mechanism of action. NF-κB has been implicated in the pathophysiology of the most aggressive forms of thyroid carcinoma, i.e. medullary and anaplastic carcinomas (Ludwig \textit{et al.} 2001, Pacifico \textit{et al.} 2004). Although no studies in xenografts were performed, bortezomib induced apoptosis in medullary and anaplastic cell lines, but not in papillary or follicular cell lines, with IC50 values well within the range of clinically achievable concentrations, showing a promising therapeutic target for aggressive thyroid cancer (Mitsiades \textit{et al.} 2006). Activation of the PI3K/Akt signalling pathway appears to be an important event in thyroid tumorigenesis and, perhaps, in tumour progression too. Pharmacological disruption of PI3-kinase activity, or disruption of Akt signalling using dominant negative cDNA expression have demonstrated beneficial effects on several cancer models \textit{in vitro}. Therefore, Akt represents an attractive target for pharmaceutical development for a variety of malignancies, including thyroid cancer (Kada \textit{et al.} 2004).

\textbf{Conclusions}

Thyroid follicular cancer biology has unique characteristics that make this malignancy a paradigm for the initiation of tumour formation. Tumour-initiating events have been identified in a high proportion of the most frequent type of thyroid cancer, PTC, and all of them converge in a single signalling pathway – the RTK/RAS/BRAF/MAPK pathway. Further studies are needed to understand the next steps in thyroid tumorigenesis as other cancer genes and/or pathways are starting to emerge. As a consequence of the development of thyroid follicular cancer genetics, genomics and epigenetics, our increasing understanding of the biology of this type of cancer is leading to new and promising approaches for the development of thyroid cancer therapies.
Acknowledgements

We are grateful to the Ministerio de Educación y Ciencia (Grants BFU 2004-03169; SAF2007-60164) and the Instituto de Salud Carlos III, FIS (grants RGDM(C03/212), PI041216, PI042374, and RD06/0020/0060) for supporting our work. G Riesco-Eizaguirre is the recipient of an MD Training Grant from FIS, Instituto de Salud Carlos III (Spain). We thank Dr Ronald Hartong for his linguistic assistance. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References


Aldred MA, Huang Y, Liyanarachchi S, Pellegrata NS, Gimm O, Jhiang S, Davuluri RV, de la Chapelle A & Eng C 2004 Papillary and follicular thyroid carcinomas show distinctly different microarray expression profiles and can be distinguished by a minimum of five genes. Journal of Clinical Oncology 22 3531–3539.


rearrangement is frequently detected in the follicular variant of papillary thyroid carcinoma. Journal of Clinical Endocrinology and Metabolism 91 213–220.


Ito T, Seyama T, Iwamoto KS, Mizuno T, Tronko ND, Komissarenko JV, Chestrovoy ED, Satow Y, Takeichi N, Dohi K et al. 1994 Activated RET oncogene in thyroid cancers of children from areas contaminated by Chernobyl accident. Lancet 344 259.


Quiros RM, Ding HG, Gattuso P, Prinz RA & Xu X 2005 Evidence that one subset of anaplastic thyroid carcinomas are derived from papillary carcinomas due to BRAF and p53 mutations. *Cancer* **103** 2261–2268.


Xing M 2005 BRAF mutation in thyroid cancer. Endocrine-Related Cancer 12 245–262.


