Abstract

Medullary thyroid carcinoma (MTC) is a rare tumour arising from neural crest-derived parafollicular C-cells. Metastatic MTC patients are incurable because the cancer does not respond to radiotherapy or chemotherapy. The REarranged during Transfection (RET) proto-oncogene plays a key role in the development of MTC. However, one-half of the sporadic MTC do not carry RET mutations. Mice models and early evidence obtained in human samples suggest that other genes, including those encoding components of the RB1 (retinoblastoma) and TP53 tumour-suppressor pathways, may be involved in MTC formation. Here, we review the data on the involvement of genes acting in the RET and RB1/TP53 pathways in MTC. Understanding genetic lesions that occur in MTC is a prerequisite to identifying molecular therapeutic targets in MTC and in improving the efficacy of RET-targeted therapies.

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Medullary thyroid carcinoma: a genetic overview

Medullary thyroid carcinoma (MTC) arises from calcitonin-producing neural crest-derived parafollicular (C) cells of the thyroid. MTC accounts for 5–8% of all thyroid cancers (Matias-Guiu et al. 2004, Schlumberger et al. 2008). MTC is sporadic in about 75% of the cases; in the remaining cases, it occurs as a component of the autosomal dominant familial multiple endocrine neoplasia type 2 (MEN 2) syndrome. MEN 2, first described by Sipple (1961), includes three disorders: MEN 2A; MEN 2B; and familial MTC. (FMTC; Marx 2005, Elisei et al. 2007, Zbuk & Eng 2007). MEN 2-associated MTC is bilateral and multicentric, and it is usually preceded by multifocal C-cell hyperplasia (CCH; Gagel & Marx 2003). REarranged during Transfection (RET) is mutated in roughly 50% of sporadic MTC and in more than 95% of MEN 2 families.

In FMTC, although the germ line RET mutation is present in all somatic cells of the affected individual, tumours are monoclonal, which suggests that other genetic alterations must occur at the somatic level and act in concert with RET mutations for the tumour to develop (Gagel & Marx 2003). Moreover, a few MEN 2 families negative for RET mutations have been described, suggesting the existence of additional loci predisposing to MEN 2 (Montero-Conde et al. 2007). Finally, about 50% of sporadic MTC do not carry RET mutations. Whether another frequently mutated gene or multiple low-frequency mutated genes occur in RET wild-type MTC samples is unknown.

In MEN 2, MTC is associated in about 50% of cases to pheochromocytoma (MEN 2A and 2B), and in 10–35% of cases to parathyroid hyperplasia or adenoma (MEN 2A; Gagel & Marx 2003). This suggests that MTC shares pathogenetic mechanisms with pheochromocytomas and parathyroid tumours. However, MTC is not a phenotype of non-MEN 2 familial forms of parathyroid tumours or pheochromocytomas (Gagel & Marx 2003, Marx 2005, Zbuk & Eng 2007). Accordingly, Prad1/ Ccnd1, Men1 and Hprt2 genes (associated to non-MEN 2 forms of parathyroid tumours; Ferris & Simental 2004) and Vhl, Nf1 and succinate dehydrogenase subunits B, C and D (SdhB, SdhC and SdhD; associated to non-MEN 2 forms of pheochromocytomas; Maher & Eng 2002, Kaelin 2008) do not seem to be mutated in MTC. SdhB, C and D were not found to be mutated in sporadic MTC (Lima et al. 2003, Cascon et al. 2005, Montani et al. 2005), although germ line SdhB and SdhD variants were over-represented in MTC samples with respect to healthy individuals (Sobrinho-Simões et al. 2008). One mutation and three monoallelic deletions were found in the Vhl gene in five familial RET mutant MTC samples, suggesting cooperation of Ret gain with Vhl loss in MTC formation (Koch et al. 2006). Intriguingly, Vhl,
NF1 and SDH gene products collaborate with RET in a common signalling pathway involved in controlling EglN3 prolyl hydroxylase-mediated neuronal cell apoptosis. In this pathway, RET (gain-of-function) and NF1 and VHL (loss-of-function) mutations lead to increased JunB transcription factor, which, in turn, blunts the expression of EglN3, thereby leading to inappropriate cell survival and tumourigenesis (Kaelin 2008). Loss of SDH activity results in higher succinate levels; this in turn triggers the survival pathway because EglN3 is feedback-inhibited by succinate. Finally, the recently discovered KIF1B tumour suppressor, which maps in a chromosomal region (1p36) frequently deleted in MTC (see below), is required for EglN3 pro-apoptotic activity (Kaelin 2008). Thus, even if not frequently mutated, these proteins should be functionally analyzed in relation to MTC formation.

In the following sections we focus on genes acting in the RET (Fig. 1) and RB/TP53 (Fig. 2) pathways.

**RET signalling pathway in MTC**

The **RET** gene in FMTC and sporadic MTC

The **RET** gene has been extensively reviewed elsewhere (Kodama et al. 2005, Kondo et al. 2006, Santoro & Carlomagno 2006, Asai et al. 2006). Therefore, here we will summarize a few key points. The **RET** protein product is a single pass transmembrane receptor with an intracellular tyrosine kinase domain (RTK = receptor tyrosine kinase) that binds glial-derived neurotrophic factor (GDNF) ligands. Ret was initially described as a *bona fide* proto-oncogene because it is activated by chromosomal aberrations in papillary thyroid carcinoma (PTC; Kondo et al. 2006, Santoro & Carlomagno 2006). Subsequently, it was found that the germ line point mutations in Ret causes MEN 2 syndrome, and similar mutations at the somatic level are the most common genetic alterations identified so far in sporadic MTC (Gagel & Marx 2003, Kouvaraki et al. 2005, Marx 2005, Zbuk & Eng 2007). Most MEN 2B patients (95% of cases) carry the M918T-mutation in RET; the remaining fraction harbours the A883F substitution or other rare mutations. In 98% of MEN 2A, the mutations affect one of the five cysteines in the extracellular cysteine-rich domain of RET. In FMTC, mutations affect either the extracellular cysteines or the intracellular domain of RET (Niccoli-Sire et al. 2001, Gagel & Marx 2003, Kouvaraki et al. 2005, Marx 2005, Elisei et al. 2007, Zbuk & Eng 2007). The genotype–phenotype correlation between the type of Ret mutation and penetrance and expressivity of the disease further supports the prime role exerted by RET mutations in FMTC (Machens & Dralle 2007). Thanks to this close correlation between a specific genetic lesion and cancer occurrence, MEN 2 is the best example in oncology of the efficacy of molecular diagnosis in mainstream clinical management. In fact, early thyroidectomy in Ret mutation carriers significantly improved their prognosis (Brandi et al. 2001, Gagel & Marx 2003, Skinner et al. 2005, Machens & Dralle 2007). Sporadic MTC, particularly more aggressive cases, also frequently (30–50% of cases) feature the M918T-RET mutation (Elisei et al. 2007, 2008). Finally, susceptibility to

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**Figure 1** Schematic representation of the RTK (receptor tyrosine kinase) signalling pathways. Potential oncoproteins are in white, whereas tumour suppressors are in grey, \( \square \) = kinases. It should be noted that only some signalling effectors are represented. RAS and AKT proteins have several effectors (like RALGDS for RAS) in addition to those represented in the figure. In addition, only some of the interactions that occur among the various proteins are represented. For instance, RTKs like RET are known to directly phosphorylate beta-catenin and STAT, besides activating them through RAS and AKT. Moreover, AKT may directly phosphorylate IKK proteins.

**Figure 2** Schematic representation of RB and p53 signalling pathways leading to cell proliferation arrest and apoptosis. Potential oncoproteins are in white, whereas tumour suppressors are in grey, \( \square \) = kinases.
sporadic MTC could be influenced by the RET polymorphisms G691S/S904S (Robledo et al. 2003, Elisei et al. 2004, Cebrian et al. 2005, Lesueur et al. 2006), however, these findings require confirmation on larger casististics (Weber & Eng 2005).

As discussed above, secondary genetic alterations at somatic level must act in concert with mutations in Ret for MTC to develop (Gagel & Marx 2003). Probably, only a small number of secondary genetic events are required in MEN 2B mutation carriers because, in these patients, the disease develops in the first few months of their life. A secondary genetic hit may target the Ret gene itself, either through duplication of the mutant allele or loss of the wild-type allele (Huang et al. 2003). Additional hits may involve chromosome deletion and amplification events, such as the deletion in chromosome lp (Mathew et al. 1987, Khosla et al. 1991, Mulligan et al. 1993, Marsh et al. 2003, Ye et al. 2008).

MTC-associated Ret-mutations convert Ret into a dominantly transforming oncogene. Extracellular cysteine MEN 2A/FMTC RET-mutants exert constitutive kinase activity consequent to ligand-independent homodimerization. In the case of mutation M918T, constitutive RET activation probably results from disruption of an auto-inhibited head-to-tail RET TK homodimer (Knowles et al. 2006). Transgenic mouse models demonstrated that RET oncogenes are able to drive MTC formation. Mice expressing RET-C634R or RET-M918T, but not wild-type RET, under the control of the calcitonin gene promoter developed MTC (Michiels et al. 1997, Acton et al. 2000). Also transgenic mice carrying RET-C634R under the control of a ubiquitous viral promoter developed MTC, suggesting that murine C-cells are highly susceptible to RET-mediated transformation (Kawai et al. 2000). However, the knock-in of the M918T mutation into mouse endogenous Ret gene caused CCH but not MTC, suggesting that, in the background of a normally expressed Ret-mutant allele, the accumulation of secondary genetic alterations is required for development of MTC (Smith-Hicks et al. 2000). Genetic background strongly affected the MTC phenotype in transgenic mice, with tumour penetrance varying from 0% in FVB/N to 98% in CBA/ca mice, which suggests that the genetic modifiers greatly affect RET-driven MTC risk (Cranston & Ponder 2003).

RET knock-down by dominant-negative mutants, ribozymes or RNAi impaired proliferation of RET-mutant MTC cell lines (Parthasarathy et al. 1999, Drosten et al. 2004). Taken together, these studies strongly implicated RET in the formation and maintenance of a subset of MTC, and provided the conceptual framework for the use of RET kinase inhibitory compounds in MTC clinical trials (Wells & Nevins 2004, Castellone et al. 2008, Schlumberger et al. 2008, Sherman 2008).

RET signalling cascade

Genetic screenings in model organisms have shown that the same phenotype can arise from alterations in any of several genes acting epistatically in common signalling cascades. Similarly, although the number of potential cancer driver genes is large, this probably reflects changes in only a few pathways. For instance, a systematic cancer genome analysis recently revealed that many mutations in colon and breast cancer cluster in genes acting in few signalling cascades (Wood et al. 2007, Sjoblom 2008). Thus, if gain-of-function RET-mutations are associated with human MTC, it is equally plausible that mutations in the genes encoding co-receptors/ligands that trigger RET activation or signalling effectors that mediate RET intracellular effects play a role in MTC. An important proof of this concept was provided by genetic analysis of PTC, another thyroid tumour type, in which RET is implicated. Indeed, it was found that most PTC cases that are negative for RET (RET/ PTC) rearrangements harbour either mutations of BRAF, an effector of the RET-initiated extracellular-signal regulated kinase (ERK) signalling cascade or, less frequently, in NTRK1, another growth factor receptor (Fig. 1: Fagin 2005, Kondo et al. 2006, Pierotti & Greco 2006).

RET is activated through the binding of four GDNF family ligands (GFL) GDNF, neurturin (NRTN), artemin (ARTN), persephin (PSPN) together with the four corresponding membrane co-receptors (GFRα1, 2, 3 and 4; Airaksinen & Saarma 2002). GFRα4, in particular, is expressed in normal C-cells and the corresponding ligand, PSPN, is required for calcitonin production by C-cells (Lindahl et al. 2001, Lindfors et al. 2006). No somatic mutation in any of the GFL/GFRA encoding genes has been reported in MTC (Marsh et al. 1997, Borrego et al. 1998), although GFL/GFRA genes map in chromosomal regions where allelic imbalances were detected in MTC (Marsh et al. 2003). Polymorphic variants of GFL/GFRA genes, particularly GFRA1 in familial (Gimm et al. 2001a, Lesueur et al. 2006) and GFRA4 in sporadic (Cebrian et al. 2005, Vanhorne et al. 2005, Ruíz-Llorente et al. 2007) MTC cases, have been reported.

Once activated, RET transmits mitogenic, survival and motogenic signals (Kodama et al. 2005, Asai et al. 2006b, Santoro & Carlomagno 2006). Two major signalling cascades, namely RAS and phosphatidylinositol 3-kinase (PI3K), are triggered by RET (Fig. 1). In turn, RAS and PI3K contribute to the activation of many signalling effectors and, as described below, they concur to the activation of nuclear factor-κB (NF-κB), signal transducer and activator of transcription (STAT) and beta-catenin. Other signalling effectors, namely SRC (Encinas et al. 2004, Iavarone et al. 2006), phospholipase
Cγ (Borrello et al. 1996, Jain et al. 2006), and RAC1/JUN NH(2)-terminal kinase (JNK; Chiariello et al. 1998, Fukuda et al. 2002, Asai et al. 2006a) are activated by RET (Fig. 1). In principle, gain-of-function of these pathways may contribute to MTC. Moreover, negative regulators of RET signalling have also been identified and, in principle, their loss-of-function may contribute to MTC formation (Fig. 1).

Hereafter, we focus on the RET pathways that have been more extensively studied in MTC. Components of these pathways may be exploited as molecular targets for MTC treatment.

**RAS pathway**

Growth-factor binding to cell surface RTKs creates docking sites for adaptor molecules that activate guanine nucleotide-exchange factors, which in turn favours GTP binding to RAS small G-proteins (KRAS, HRAS and NRAS; Schubbert et al. 2007). Intrinsic RAS-GTPase activity terminates signalling, a reaction that is accelerated thousands of fold by GTPase-activating proteins such as neurofibromin (NF1; Fig. 1; Schubbert et al. 2007). Once activated, RAS stimulates numerous intracellular transducers, including RAF, P13K and Ral guanine nucleotide-dissociation stimulator, to regulate proliferation, survival and differentiation (Fig. 1; Halilovic & Solit 2008). The RAS–RAF–MEK–ERK cascade is the best characterized RAS-effector pathway. There are three RAF serine/threonine kinases (ARAF, BRAF and CRAF) that activate the MEK (MAP2K1/MAP2K2)–ERK (MAPK3/MAPK1) kinase cascade. ERK, in turn, stimulates gene transcription by directly phosphorylating transcription factors or by targeting intracellular kinases like p90RSK (Fig. 1; Schubbett et al. 2007). Negative regulators attenuate RAS signalling at various levels of the signalling cascade (Fig. 1; see below).

RAS genes are most commonly activated by point mutations in cancer. Alternatively, the RAS pathway can be triggered indirectly by loss of the negative regulator NF1, by upstream activation of cell surface RTKs or PTPN11 (which encodes the Src homology-2-containing protein tyrosine phosphatases-2 (SHP-2)) or by downstream activation of RAS signalling effectors (Fig. 1; Wellbrock et al. 2004, Halilovic & Solit 2008). This paradigm applies to thyroid carcinoma of follicular cell lineage, where RET gene rearrangements are prevalent in PTC, RAS-mutations in follicular carcinoma and in follicular-variant PTC, and BRAF-mutations in PTC and anaplastic carcinoma (Kondo et al. 2006). Sequencing analysis of all three RAS family members did not reveal any mutation in about 30 MTC samples (Moley et al. 1991, Horie et al. 1995, Bockhorn et al. 2000). Similarly, no BRAF-mutation was found in 65 MTC samples (Xing 2005). Taken together, these findings excluded that RAS/BRAF gene mutations exert a prominent role in MTC formation. However, a recent study led to a different conclusion by showing 41% KRAS-mutations and 68% BRAF-mutations in MTC samples (Goutas et al. 2008).

The degree and duration of activation dictate the final biological outcome of RAS signalling. For example, in PC12 pheochromocytoma cells, transient RAS activation stimulates proliferation, whereas sustained RAS activation induces differentiation (Schubbert et al. 2007). Similarly, oncogenic HRAS and CRAF or RAF1 alleles decreased MTC cell proliferation and increased calcitonin gene expression (Nakagawa et al. 1987, Carson-Walter et al. 1998). Such a pro-differentiating effect of constitutive RAS–RAF signalling may explain why mutations in these genes are unlikely to occur in MTC. In this context, NRAS exerted a protective effect against MTC formation as shown by the finding that NRAS deletion increased MTC formation in RB1-knock-out mice (see also below; Takahashi et al. 2006). However, the role of RAS signalling in MTC cells is probably complex and different components of the RAS family may exert different effects. In fact, when targeted to C-cells, an oncogenic HRAS mutant caused MTC in transgenic mice (Johnston et al. 1998). Similarly, Moloney murine sarcoma virus oncogene (MOS) another oncogene that potently activates ERK induced MTC and pheochromocytoma in transgenic mice (Schulz et al. 1992). With the caveat that findings obtained in artificial animal models should be interpreted with caution, it is conceivable that RAS signalling along the ERK cascade is involved in mitogenic signalling in MTC cells. In this context, it is noteworthy that inhibition of the ERK pathway reduced proliferation of a RET-mutant MTC cell line (Zatelli et al. 2005). Inhibitors of MEK are currently undergoing clinical experimentation in thyroid cancer patients (Sherman 2008).

**PI3K pathway**

Class I PI3K are constituted by a regulatory (p85α, p55γ, p50α, p85β, p55γ) and a catalytic (p110α, p110β, p110δ) subunit. Upon recruitment to the plasma membrane by activated RTK or RAS, class I PI3K phosphorylates phosphatidylinositol-4, 5-bisphosphate to generate phosphatidylinositol-3, 4, 5-trisphosphate (PIP3; Fig. 1). PIP3, in turn, activates downstream molecules such as the RAC small GTPase, 3-phosphoinositide-dependent protein kinase 1 (PKD1), and the AKT (also known as PKB) serine/threonine kinase (Yuan & Cantley 2008). The lipid phosphatase, phosphatase and tensin homologue deleted on chromosome 10 (PTEN) antagonizes this cascade by dephosphorylating PIP3 (Salmena et al. 2008). Besides, buffering the PI3K pathway, PTEN also
exerts phosphatase-independent nuclear functions that may contribute to the potent oncogenic effect resulting from its inactivation in tumours (Salmena et al. 2008). AKT phosphorylates and inactivates pro-apoptotic transcription factors of the forkhead-box class O (FOXO) family, the cell-cycle inhibitor CDKN1B (p27Kip1), and the GSK3B kinase, thereby releasing beta-catenin from the inhibitory effects of GSK3B (see below; Fig. 1; Yuan & Cantley 2008). In addition, AKT stimulates the serine/threonine kinase mammalian target of rapamycin (mTOR; Fig. 1). mTOR is associated with two complexes: the rapamycin-sensitive TORC1 complex (that phosphorylates S6K to regulate protein translation); and the rapamycin-insensitive TORC2 (which is the PDK2 activity that controls serine 473 phosphorylation of AKT itself; Bjornsti & Houghton 2004). TORC1 also contributes to NF-κB activation (see below; Fig. 1).

The PI3K–AKT–mTOR cascade is important in tumourigenesis because of its ability to promote growth (cell size) and proliferation (cell number) and to prevent cell death. Mutations in major nodes of this cascade are prevalent in human cancer and include gain-of-function mutations and amplification of the genes encoding the catalytic subunit p110α of PI3K (PIK3CA) and AKT (Zbuk & Eng 2007, Yuan & Cantley 2008). Mutations in this pathway are very frequent, for instance, in breast and colon cancer (Wood et al. 2007). Germ line inactivating mutations of PTEN cause autosomal dominant hamartoma syndromes, and somatic PTEN inactivation by deletion is very frequent (up to 30–50%) in sporadic tumours (Zbuk & Eng 2007, Paes & Ringel 2008). Many studies have demonstrated that the PI3K–AKT system plays a key role in RET signalling (Segoufin-Cariou & Billaud 2000, Kodama et al. 2005, Asai et al. 2006b). However, no systematic genetic analysis of PI3K pathway components has been reported so far in MTC. PIK3CA gene amplification, which frequently occurs in aggressive tumours of thyroid cells of follicular lineage, was not detected in 13 MTC samples (Wu et al. 2005). PTEN analysis in MTC has so far been limited to promoter methylation assessment, and no methylation was detected in a small MTC set (Schagdarsurengin et al. 2006). However, CCH and MTC occur in PTENheterozygous mice, particularly when crossed with mice knock-out for CDKN2C (encoding the p18Ink4c cell-cycle inhibitor; see below; Bai et al. 2006). Thus, as discussed for RAS, the PI3K–AKT cascade, even though infrequently mutated, may play a role in MTC. Accordingly, in vitro chemical PI3K inhibition reduced MTC cell proliferation and survival, which indicates that this pathway could be a molecular target in MTC treatment (Kunnimalaiyaan et al. 2006a). Given its central role in PI3K–AKT signalling, and the availability of potent and selective inhibitors (everolimus, temsirolimus) derived from rapamycin (sirolimus), mTOR is one of the most appealing therapeutic targets in this pathway (Bjornsti & Houghton 2004).

NF-κB

The NF-κB family includes five transcription factors named NF-κB1 (p50), NF-κB2 (p52), Rel, RelA (p65) and RelB. NF-κB activates transcription of genes associated with cell proliferation, angiogenesis, metastasis, and inflammation and suppression of apoptosis (Baud & Karin 2009). NF-κB proteins are rendered inactive in non-stimulated cells through binding to inhibitors, known as the IκB (IκB α, β, ε) proteins. Activation of most forms of NF-κB, especially the most common form (the p50/RelA dimer), depends on phosphorylation-induced ubiquitination of IκB that is mediated by the IκB kinase (IKK) complex (CHUK, IKBKB, IKBKG or NEMO; Baud & Karin 2009). Thus, NF-κB is activated by different membrane receptors as well as by BRAF that directly associates with IKK (Encinas et al. 2008) and by PI3K/AKT that mediates an mTOR/IKK interaction (Dan et al. 2008; Fig. 1). RET stimulates IKK phosphorylation and NF-κB activation, thus contributing to MTC cell survival (Ludwig et al. 2001, Encinas et al. 2008).

Recent studies have found mutations that directly target NF-κB pathway components in human cancer (Wood et al. 2007). A genetic analysis of the NF-κB pathway in MTC has not yet been reported. Histochemical analysis of MTC tissue samples revealed that many proteins of the NF-κB family, particularly p65, p52 and c-Rel, are localized in the nucleus (Gallel et al. 2008). NF-κB inhibitors, particularly IKK inhibitors, are being exploited in cancer therapy (Baud & Karin 2009). Moreover, inhibitors of the 26S proteasome, such as bortezomib (Velcade), that prevent IκB degradation and NF-κB nuclear translocation, exerted cytotoxic effects in MTC cells (Mitsiades et al. 2006b).

Beta-catenin (CTNNB1)

Beta-catenin, which is encoded by the CTNNB1 gene, plays an important role in cellular adhesion by associating with E-cadherin and alpha-catenin. Upon disassembly of the membrane complex, beta-catenin migrates into the nucleus where it acts as a co-activator of TCF/LEF (T-cell factor/lymphoid-enhancing factor) transcriptional factors (Brembeck et al. 2006). RET stimulates beta-catenin activation via direct phosphorylation on Y654 and via PI3K/AKT- and RAS/ERK-mediated inhibition of GSK3B (Fig. 1; Cassinelli et al. 2009, Gujral et al. 2008, Castellone et al. 2009). Although an analysis of the CTNNB1 gene in MTC has not yet
been reported, MTC samples from human patients and RET(M918T) transgenic mice showed nuclear beta-catenin accumulation (Gujrjal et al. 2008).

STAT

STAT transcription factors are activated in response to cytokines and growth factors. Cytokines activate STAT through JAK tyrosine kinases, whereas RTKs can phosphorylate STAT directly. The JAK–STAT pathway has been implicated in several neoplastic diseases, particularly myeloproliferative disorders (Levine & Gilliland 2008). Oncogenic RET mutants induce serine phosphorylation through the RAS pathway, and tyrosine phosphorylation of STAT3 (Fig. 1; Plaza Menacho et al. 2005, 2007). Moreover, activated STAT3 was identified in the nucleus of cells from MTC samples (Plaza Menacho et al. 2005).

Negative regulators of RET signalling

Several proteins function as feedback regulators to attenuate RTK signalling and, intriguingly, the corresponding genes are often downregulated in diverse tumour types (van Staveren et al. 2006, Amit et al. 2007). Negative regulators of RET signalling have been identified. However, also in this case, no systematic analysis of genetic alterations in MTC has yet been reported. Below, we briefly discuss the effects exerted by tyrosine phosphatases (LAR, PTPRJ, SHP-1), ERK dual-specificity phosphatases (DUSP) and RAS–BRAF signalling inhibitors (SPRY) on RET signalling.

Tyrosine phosphatases dephosphorylate RET and attenuate RET signalling; theoretically, their loss could promote MTC formation (Fig. 1). Leukocyte common antigen-related (LAR) phosphatase (also called ‘PTPRF’, protein tyrosine phosphatase, receptor type, F) is a receptor tyrosine phosphatase that maps on a region of chromosome 1 (1p) that is frequently lost in MTC (Mathew et al. 1987, Mulligan et al. 1993). LAR forms stable complexes with RET and dephosphorylates RET cysteine mutants (but not RET-M918T) thereby blunting cell proliferation (Qiao et al. 2001). Similarly, the receptor-protein tyrosine phosphatase J (PTPRJ) binds and de-phosphorylates RET cysteine mutants and thus impairs their transforming effect (Iervolino et al. 2006). The SHP-1, SHP-2 are non-transmembrane phosphotyrosine phosphatases (PTPN6 and PTPN11 respectively). While SHP-2 functions as a positive RTK signal transducer and stimulates downstream RET signalling along the RAS cascade (D’Alessio et al. 2003), SHP-1 serves as a negative regulator of signalling systems. SHP-1 associates with RET, restrains RET autophosphorylation, and inhibits MTC cell proliferation (Hennige et al. 2001, Incoronato et al. 2004, Zatelli et al. 2005). Intriguingly, SHP-1 is involved in the cytostatic effects of somatostatin in MTC cells (Zatelli et al. 2005).

Activated ERKs are inactivated through dephosphorylation of threonine and/or tyrosine residues within the activation loop. The DUSP, also called ‘MAP kinase phosphatases’ (MKP), carries out this function (Fig. 1; Kondoh & Nishida 2007). Intriguingly, MKPs/DUSPs are rapidly induced upon growth factor signalling, and functions as feedback regulators of the pathway (Amit et al. 2007). RET-mediated signalling increased MKP-3 levels (Colucci-D’Amato et al. 2000). In principle, a loss-of-function of MKPs may favour RET signalling along the ERK cascade. However, it should be noted that the pro-mitogenic and anti-mitogenic effects of MKPs/DUSPs may vary depending on the specific complement of MAPK family members they dephosphorylate. For instance, DUSP4/MKP-2, which dephosphorylates not only p42/44 MAPK (ERK) but also p38MAPK and JNK, exerts a positive (rather than a negative) role in RET-mediated tumourigenesis and it is upregulated in MTC samples (Hasegawa et al. 2008).

Sprouty (SPRY) and Spred proteins are evolutionarily conserved inhibitors of signalling that act by blocking RAS–RAF interaction and ERK activation. The expression of SPRY family members is induced by RET, and SPRY2 blunted RET–ERK signalling (Ishida et al. 2007). Intriguingly, genetic ablation of SPRY2 led to enteric neuronal hyperplasia by promoting RET signalling (Taketomi et al. 2005). Similarly, SPRY1-deficient mice had kidney defects because of RET hypersignalling (Basson et al. 2005). SPRY/SPRED downregulation has been reported in several human cancers (Lo et al. 2006). Germ line loss-of-function mutations in SPRED1 caused a neurofibromatosis 1-like syndrome (Brems et al. 2007). Thus, a loss of SPRY/SPRED family members in C-cells can, in principle, favour MTC formation.

Other growth factor receptors

It is conceivable that other RTKs, besides RET, are involved in MTC. This point is of great topical interest because tyrosine kinase inhibitors are now being tested in MTC patients (Castellone et al. 2008, Sherman 2008). Proliferation of cultured MTC cells is stimulated by insulin-like growth factor 1 (IGF1) and inhibited by compounds targeting IGF1-R (Yang et al. 1992, Misiades et al. 2004). NTRKs, which are tyrosine kinase receptors for growth factors of the nerve growth factor family, have been studied in MTC because, like RET, they exert neurotrophic effects and are involved in PTC (NTRK1 rearrangements; Pierotti & Greco 2006). Moreover, there is functional evidence that NTRK1–RET signalling is involved in neuronal cell survival (Tsui-Pierchala et al. 2002, Luo et al. 2007, Pierchala et al. 2007). Although no
mutations have been found in NTRK1, 2 and 3 (Gimm et al. 1999, 2001b), NTRK2 expression was reduced, whereas NTRK3 expression was increased in MTC (McGregor et al. 1999). Moreover, NTRK2 expression impaired the tumourigenicity of MTC cells (McGregor et al. 1999). Interaction between epidermal growth factor receptor (EGFR) and RET was recently found to mediate EGFR-dependent RET activation (Croyle et al. 2008). Phosphorylated EGFR has been identified in MTC cells (Gotla et al. 2008). It is noteworthy that Vandetanib, a RET kinase inhibitor currently being investigated in MTC patients, is also an EGFR inhibitor (Carlomagno et al. 2002). No mutation in EGFR was found in small MTC sample sets (Mitsiades et al. 2006a, Cerrato & Santoro unpublished). Fibroblast growth factor receptor-4 (FGFR4) is expressed in aggressive thyroid tumour types and MTC cells. Molecular targeting of FGFR-4 with an ATP-competitive inhibitor prevented the growth and reduced the tumourigenesis of MTC cells (Ezzat et al. 2005).

Finally, membrane receptors of families other than the RTK family have been implicated in MTC. NOTCH1 is a multifunctional transmembrane receptor that regulates cell differentiation, development, proliferation and survival. Binding of several ligands promotes proteolytic cleavage events, which result in the release of the NOTCH1 intracellular domain that, in turn, translocates to the nucleus and activates transcription of various target genes. NOTCH1 is a negative regulator of ASH1 (achaete-scute homolog-1, called ‘MASH1’ in rodents), which is a highly conserved basic helix-loop-helix transcription factor that is critical for C-cell development (Lanigan et al. 1998). Interestingly, MTC expresses ASH1 but not NOTCH1, and NOTCH1 expression arrested proliferation of MTC cells (Kunnimalaiyaan et al. 2006b). The prolactin receptor (PRLR) belongs to the cytokine receptor family and activates the JAK–STAT pathway. Unexpectedly, PRLR-null mice developed MTC at a high frequency, thereby suggesting that PRLR suppresses MTC formation at least in mice (Kedzia et al. 2005).

**Tumour suppressors of the RB1 and TP53 pathways in MTC**

The tumour-suppressor genes RB1 (retinoblastoma: pRB protein) and TP53 (p53 protein) are frequently mutated in human cancer, and several lines of evidence indicate that both pathways must be inactivated in cancer to overcome senescence or apoptosis (Hahn & Weinberg 2002). RB1 is the prototypic member of the class of tumour suppressors known as ‘gatekeepers’, which control tumour growth in a cell-autonomous manner. This mainly depends on pRB’s ability to repress the effect exerted by the E2F/DP family of transcription factors, namely, stimulation of cell-cycle progression or apoptosis (Fig. 2; Hahn & Weinberg 2002). Binding of the pRB protein to E2F/DP transcription factors is high when pRB is hypophosphorylated in G1, and low when pRB is hyperphosphorylated in S and G2 phases. pRB is phosphorylated sequentially by D-, E- and A-type cyclin-mediated CDK activity. In turn, CDKs are negatively regulated by CDK inhibitors (CKI) of the INK4 (p16INK4A, p15INK4B, p18INK4C, p19INK4D) and CIP/KIP (p21CIP1, p27KIP1, p57KIP2) families (Fig. 2). Tethering of pRB to E2F target genes results in cell-cycle arrest (Trimarchi & Lees 2002). There are multiple interactions between the pRB and the p53 pathways (Fig. 2). On one hand, by stimulating transcription of the p21CIP1 (CDKN1A) cell-cycle inhibitor, p53 obstructs the activity of cyclin E/CDK complexes, thereby reducing pRB phosphorylation and, consequently, E2F activity. On the other hand, loss-of-function of pRB releases not only the pro-mitogenic but also pro-apoptotic activity of E2F transcription factors. The final outcome may depend on TP53 genetic status because E2F-mediated apoptosis is dependent on the upregulation of p14ARF (CDKN2B) that in turn stabilizes p53. Therefore, in cancer, RB1 and TP53 are often concurrently mutated (Hahn & Weinberg 2002).

There is extensive genetic evidence in rodents that the pRB and p53 pathways are involved in MTC. RB1-deficient mice developed MTC (Harrison et al. 1995). Conditional RB1 inactivation also induced highly aggressive MTC in mice (Kucherlapati et al. 2006). Loss of TP53 further increased MTC formation in RB1-deficient mice (Williams et al. 1994, Harvey et al. 1995). E2F family transcription factors exerted a dual role in MTC formation. Genetic deletion of E2F1 or E2F4 reduced MTC formation in RB1-deficient mice (Yamasaki et al. 1998, Lee et al. 2002). Instead, deletion of E2F3 further increased the incidence and aggressiveness of MTC (Ziebold et al. 2003).

Interestingly, MTC from RB1/TP53-deficient mice acquired somatic cysteine mutations in RET that closely resemble activating mutations observed in human MTC. This suggested that murine MTC requires mutational dysregulation within both the RET and nuclear tumour suppressor gene pathways (Coxon et al. 1998). High grade MTC were observed in mice simultaneously lacking RB1 and CDKN1B (that codes for the p27Kip1 cell-cycle inhibitor; Park et al. 1999). Interestingly, germ line mutation in CDKN1B predisposed rats to a multiple endocrine neoplasia syndrome featuring MTC formation (Pellegata et al. 2006). In transgenic mice, the loss of two CDKIs, CDKN1B and CDKN2C (coding for the p18INK4C cell-cycle inhibitor), led to accelerated MTC formation (Franklin et al. 2000, Joshi et al. 2007). CDKN2C deficiency also accelerated MTC formation in PTEN-deficient mice (Bai et al. 2006). Finally, transgenic mice expressing
oncogenic \textit{RET} crossed with mice lacking \textit{CDKN2C} developed MTC at a higher incidence and sooner than their single mutant littermates (van Veelen et al. 2008).

Taken together, these studies provide robust evidence that, in rodents, disruption of the \textit{RB1} and \textit{TP53} pathways predisposes to MTC formation. However, mice models may not faithfully mimic the human situation, and the tumour spectrum may significantly differ in the two species. A prominent example of this concept is provided by the phenotype of \textit{RB1}-deficient mice. In humans, loss of the \textit{RB1} gene is associated with the development of retinoblastoma and osteosarcoma and, later in life, small-cell lung carcinoma, whereas \textit{RB1}-deleted mice do not develop these types of tumours, and develop retinoblastoma only when the \textit{RB1}-related \textit{RBL1} gene is concurrently deleted (Rangarajan & Weinberg 2003). Previous studies did not find \textit{TP53}-mutations in sets of 9 (Yoshimoto et al. 1992) and 22 (Herfarth et al. 1997) MTC samples. More recent studies identified a high prevalence of \textit{TP53} mutations (Pavelić et al. 2006) and deletions in MTC (Sheikh et al. 2004). Very recently, about 10\% of MTCs were found to carry loss-of-function mutations in \textit{CDKN2C} (van Veelen et al. 2009); however, we did not find any \textit{CDKN2C}mutation in 15 MTC samples (Cerrato & Santoro unpublished). A systematic analysis of the genes in the \textit{RB1} and \textit{TP53} pathways in human samples will help to clarify their role in MTC formation. Given the role played by these tumour-suppressor pathways in the response of tumours to therapy, this information might be important for the analysis of data from the ongoing MTC trials involving the use of targeted agents.

\textbf{Conclusions}

The identification of \textit{RET} mutations has revolutionized the medical treatment of patients with FMTC. Twenty-five years after this seminal discovery, no other genetic lesion has been consistently associated with MTC formation. Studies of the \textit{RET} pathway and mouse models of MTC formation are generating an ever-growing list of genes, including the recently described \textit{CDKN2C} gene (p18INK4c cell-cycle inhibitor), that could play a role in MTC. Biochemical data also indicate that these pathways play a role in MTC formation. A thorough analysis of these genes has not yet been performed, and the results of the few studies available, conducted, moreover, on a limited number of samples, are often conflicting. An unbiased genome-wide analysis of sequence variations, copy gains and losses will probably provide groundbreaking information as has occurred for various tumour types (Sjöblom 2008). It is expected that identification of lesions in genes other than \textit{RET} will clarify the biology of MTC and foster the development of targeted therapeutic approaches. In any event, the data acquired in recent years about the signalling mechanisms operating in MTC show that molecular targeting of pathways like the RAS/ERK, PI3K/AKT and NF-\kappa B pathways is a plausible therapeutic approach for this cancer.

\textbf{Declaration of interest}

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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