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Molecular genetics of medullary thyroid carcinoma: the quest for novel therapeutic targets

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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 *RET* proto-oncogene plays a key role in the development of MTC. However, one half of 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 *RB* (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 *RB/TP53* pathways in MTC. Understanding genetic lesions that occur in MTC is a prerequisite to identifying molecular therapeutic targets in MTC and to improving the efficacy of RET-targeted therapies.

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% to 8% of all thyroid cancers (Matias-Guiu *et al*. 2004; Schlumberger *et al*. 2008). MTC is sporadic in about 75% of 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 J.H. Sipple (Sipple 1961), includes three disorders: MEN 2A, MEN 2B and familial MTC (FMTC) (Marx 2005; Zbuk & Eng 2007; Elisei *et al*. 2007). MEN 2-associated MTC is bilateral and multicentric, and it is usually preceded by multifocal C-cell hyperplasia (CCH) (Gagel & Marx 2003). *RET* is mutated in roughly 50% of sporadic MTC and in more than 95% of MEN 2 families.

In familial MTC, 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 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 (Marx 2005; Zbuk & Eng 2007; Gagel & Marx 2003). Accordingly, *PRAD1/CCND1, MEN1* and *HPRT2* genes (associated to non-MEN 2 forms of parathyroid tumours) (Ferris & Simental, 2002) and *VHL*, *NF1* and *SDHB, SDHC* and *SDHD (Succinate Dehydrogenase Subunit B, C* and *D*) (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 (Montani *et al*. 2005; Cascon *et al*. 2005; Lima *et al*. 2003), although germline *SDHB* and *SDHD* variants were over-represented in MTC samples with respect to healthy individuals (Sobrinho-Simões *et al*. 2008). One mutation and three mono allelic deletions were found in the *VHL* gene in 5 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 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 familial and sporadic MTC — The *RET* (REarranged during Transfection) gene has been extensively reviewed elsewhere (Kodama *et al*. 2005; Santoro & Carlomagno 2006; Kondo *et al*. 2006; Asai *et al*. 2006a). 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) (Santoro & Carlomagno 2006; Kondo *et al*. 2006). Subsequently, it was found that germline point mutations in *RET* cause MEN 2 syndromes, and similar mutations at somatic level are the most common genetic alterations identified so far in sporadic MTC (Kouvaraki *et al.* 2005; Gagel & Marx 2003; Zbuk & Eng 2007; Marx 2005). 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 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* (Kouvaraki *et al.* 2005; Gagel & Marx 2003; Zbuk & Eng 2007; Marx 2005; Niccoli-Sire *et al.* 2001; Elisei *et al*. 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 familial MTC (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* mutations 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; Elisei *et al.* 2008). Finally, susceptibility to 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 casistics (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 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 homo-dimer (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 C-cell hyperplasia 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 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; Schlumberger *et al*. 2008; Sherman 2008; Castellone *et al*. 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; Sjöblom 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 papillary thyroid carcinoma (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 ERK signalling cascade or, less frequently, in *NTRK1*, another growth factor receptor (Fig. 1) (Fagin 2005; Pierotti & Greco 2006; Kondo *et al*. 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/GFRα* encoding genes has been reported in MTC (Marsh *et al.* 1997; Borrego *et al*. 1998), although *GFL/GFRα* genes map in chromosomal regions where allelic imbalances were detected in MTC (Marsh *et al*. 2003). Polymorphic variants of *GFL/GFRα* genes, particularly *GFRα1* in familial (Gimm *et al*. 2001a; Lesueur *et al*. 2006) and *GFRα4* in sporadic (Vanhorne *et al*. 2005; Cebrian *et al*. 2005; Ruiz-Llorente *et al*. 2007) MTC cases, have been reported.

Once activated, RET transmits mitogenic, survival and motogenic signals (Kodama *et al*. 2005; Santoro & Carlomagno 2006; Asai *et al*. 2006a). 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 NF-κB (nuclear factor-κB), STAT (Signal Transducer and Activator of Transcription) and β-catenin. Other signalling effectors, namely SRC (Encinas *et al*. 2004; Iavarone *et al*.

2006), phospholipase Cγ (Borrello et al. 2002; Jain *et al*. 2006), and RAC1/JUN NH(2)-terminal kinase (JNK) (Chiariello *et al*. 1998; Fukuda *et al*. 2002; Asai *et al*. 2006b) 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 (GAPs) such as neurofibromin (NF1) (Fig. 1) (Schubbert *et al*. 2007). Once activated, RAS stimulates numerous intracellular transducers, including RAF, phosphatidylinositol 3-kinase (PI3K) and Ral guanine nucleotide-dissociation stimulator (RALGDS), 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 (MEK1/MEK2) -> ERK (ERK1/2) kinase cascade. ERK (extracellular-signal regulated kinase), in turn, stimulates gene transcription by directly phosphorylating transcription factors or by targeting intracellular kinases like p90RSK (Fig. 1) (Schubbert *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 SHP-2 tyrosine-phosphatase) 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 (FTC) and in follicular-variant PTC (FV-PTC), and *BRAF* mutations in PTC and anaplastic carcinoma (ATC) (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* 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, *MOS* (Moloney murine sarcoma virus oncogene), 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 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).

Phosphatidylinositol 3-kinase (PI3K) pathway — Class I PI3K are constituted by a regulatory (p85α, p55α, 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 (PIP2) to generate phosphatidylinositol-3,4,5-triphosphate (PIP3) (Fig. 1). PIP3, in turn, activates downstream molecules such as the RAC small GTPase, 3-phosphoinositide-dependent protein kinase 1 (PDK1), and the AKT (also known as PKB) serine/treonine kinase (Yuan & Cantley 2008). The lipid phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) 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 FOXO (Forkhead-Box Class O) family, the cell cycle inhibitor p27Kip1, and the GSK3β kinase, thereby releasing β-catenin from the inhibitory effects of GSK3β (see below) (Fig. 1) (Yuan & Cantley 2008). In addition, AKT stimulates the serine/threonine kinase mTOR (mammalian target of rapamycin) (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 (Segouffin-Cariou *et al*. 2000; Kodama *et al*. 2005; Asai *et al*. 2006a). 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, C-cell hyperplasia and MTC occur in *PTEN* heterozygous mice, particularly when crossed with mice knocked-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 (nuclear factor-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 (IKK-α, IKK-β, IKK-γ 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-β 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*. 2006a).

β-Catenin (CTNNB1) — β-Catenin, which is encoded by the *CTNNB1* gene, plays an important role in cellular adhesion by associating with E-cadherin and α-catenin. Upon disassembling of the membrane complex, β-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 β-catenin activation *via* direct phosphorylation on Y654 and *via* PI3K/AKT and RAS/ERK-mediated inhibition of GSK3β (Fig. 1) (Gujral *et al*. 2008; Cassinelli *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 β-catenin accumulation (Gujral *et al*. 2008).

STAT (Signal Transducer and Activator of Transcription) — 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 de-phosphorylate RET and attenuate RET signalling; theoretically, their loss could promote MTC formation (Fig. 1). LAR (Leukocyte common Antigen-Related) 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 de-phosphorylates 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 Src homology-2-containing protein tyrosine phosphatases-1 and -2 (SHP-1, SHP-2) are non-transmembrane phosphotyrosine phosphatases. 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 to 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 dual-specificity phosphatases (DUSP), also called "MAP kinase phosphatases" (MKP), carry out this function (Fig. 1) (Kondoh & Nishida 2007). Intriguingly, MKPs/DUSPs are rapidly induced upon growth factor signalling, and function 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 de-phosphorylate. 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 up-regulated 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). Germline 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 (TKIs) are now being tested in MTC patients (Castellone *et al*. 2008; Sherman 2008). Proliferation of cultured MTC cells is stimulated by IGF-I (insulin-like growth factor-I) and inhibited by compounds targeting IGF-I-R (Yang *et al*. 1992; Mitsiades *et al*. 2004). NTRKs, which are tyrosine kinase receptors for growth factors of the NGF (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 EGFR (epidermal growth factor receptor) and RET was recently found to mediate EGFR-dependent RET activation (Croyle *et al*. 2008). Phosphorylated EGFR has been identified in MTC cells (Gorla *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*. 2006b; Cerrato & Santoro unpublished).

Fibroblast growth factor receptor-4 (FGFR-4) 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 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, germline 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 *RET* crossed with mice lacking *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 *RB1* and *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 *RB1*-deficient mice. In humans, loss of the *RB1* gene is associated with the development of retinoblastoma and osteosarcoma and, later in life, small-cell lung carcinoma, whereas *RB1*-deleted mice do not develop these types of tumours, and develop retinoblastoma only when the *RB1*-related *RBL1* gene is concurrently deleted (Rangarajan *et al*. 2003). Early studies did not find *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 *TP53* mutations (Pavelić *et al*. 2006) and deletions in MTC (Sheikh *et al*. 2004). Very recently, about 10% MTC were found to carry loss-of-function mutations in *CDKN2C* (van Veelen *et al*. 2009); however, we did not find any *CDKN2C* mutation in 15 *MTC* samples (Cerrato & Santoro unpublished). A systematic analysis of the genes in the *RB1* and *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.

Conclusions

The identification of *RET* mutations has revolutionized the medical treatment of patients with familial MTC. Twenty-five years after this seminal discovery, no other genetic lesion has been consistently associated with MTC formation. Studies of the *RET* pathway and mouse models of MTC formation are generating an ever-growing list of genes, including the recently described *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 *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-kB pathways is a plausible therapeutic approach for this cancer.

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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Figure Legends

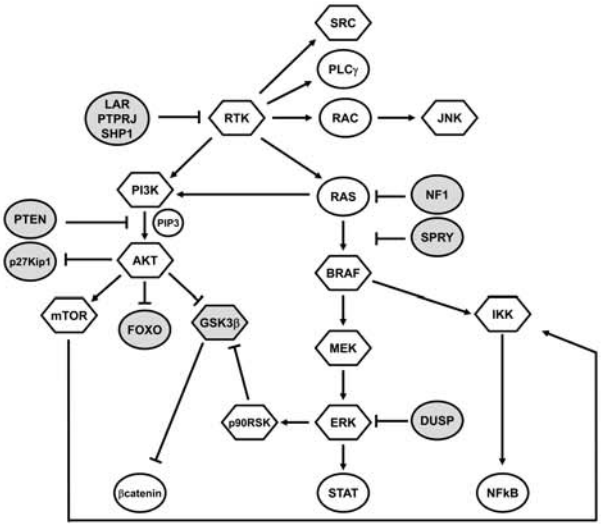
Figure 1

Schematic representation of the RTK (receptor tyrosine kinase) signalling pathways. Potential oncoproteins are in white, whereas tumour suppressors are in gray. ⬬ = 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 β-catenin and STAT, besides activating them through RAS and AKT. Moreover, AKT may directly phosphorylate IKK proteins.

Fig. 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. ⬬ = kinases.

Figure 1


SRC
PLCγ
LAR
PTPRJ
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RTK
RAC
JNK
PI3K
RAS
NF1
PTEN
PIP3
SPRY
p27Kip1
AKT
BRAF
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IKK
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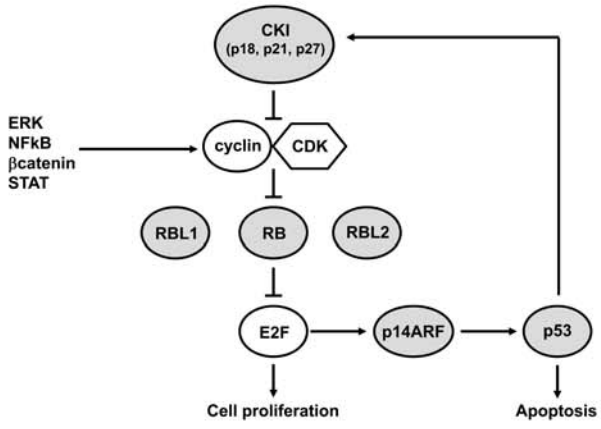
ERK
DUSP
βcatenin
STAT
NFkB

Figure 2


CKI
(p18, p21, p27)
ERK
NFkB
βcatenin
STAT
cyclin
CDK
RBL1
RB
RBL2
E2F
p14ARF
p53
Cell proliferation
Apoptosis