Medullary Thyroid Cancer: A Promising Model for Targeted Therapy

F. Torino[1], R.M. Paragliola[2], A. Barnabei[3] and S.M. Corsello*,[2]

[1]*Medical Oncology Division, "San Filippo Neri" Hospital, Rome, Italy*

[2]*Endocrinology Unit, Catholic University, Rome, Italy*

[3]*Endocrinology Unit, Regina Elena Institute, Rome, Italy*

Abstract: In recent years, the clinical validation of molecular targeted therapies inhibiting the action of pathogenic tyrosine kinase (TK) has been one of the most exciting developments in cancer research. In this context, medullary thyroid carcinoma (MTC) represents a promising model. It is well known that in MTC, the RET receptor TK and its signal transduction pathways, lead to subsequent neoplastic transformation. Several strategies aimed at blocking the activation and signaling of RET have been preclinically tested. The most advanced results have been obtained by competitive inhibition of RET-TK activity by tyrosine kinases inhibitors (TKI). However, although the inhibition of the RET pathway is actually one of the most studied for therapeutic purposes, other signal transduction pathways have been recognized to contribute to the growth and functional activity of MTC and are considered attractive therapeutic targets.

To date, surgery represents the only curative treatment of MTC. Despite promising initial results, studies on targeted agents are in early stages and several issues regarding preclinical evaluations and clinical trials of new targeted agents in MTC are still unresolved. Now, available mouse models bearing mutations of *RET* or other genes, which spontaneously develop MTC, promise to improve preclinical evaluation of activity of targeted compounds. Furthermore, the rarity of the disease and the number of patients available for enrolment may lessen the relevance of clinical trials. A major effort needs to be made by endocrinologists and oncologists to refer their patients for multi-institutional trials in order to optimize them, perform translational studies and expedite the availability of novel beneficial selective therapies.

Keywords: Tyrosine kinase inhibitors, medullary thyroid carcinoma, multiple endocrine neoplasia, RET, targeted therapy, vandetanib, sunitinib, sorafenib.

INTRODUCTION

Thyroid carcinomas account for approximately 1% of all human malignancies, which represents 3 in every 100,000 people in Europe and the U.S. [1]. Medullary thyroid carcinoma (MTC) arises from calcitonin (CTN)-producing neural crest derived parafollicular C cells of the thyroid and currently accounts for 5%–8% of all thyroid cancers [2]. Prognosis of patients affected by MTC mainly depends on the stage of the tumor at the time of diagnosis, with an estimated mean 10-year survival of 60–70% [3, 4]. The clinical course of MTC varies from an extremely indolent tumor and survival of several years, to an aggressive variant associated with a high mortality rate. MTC is sporadic in about 75% of cases; in about 25% it is a component of the inherited type 2 multiple endocrine neoplasia (MEN-2) syndromes [5]. MEN-2 are autosomal dominant inherited diseases characterized by a strong predisposition to develop endocrine tumors (Table **1**).

The three distinct clinical varieties, MEN-2A, MEN-2B and familial MTC (FMTC) are all characterized by the occurrence of MTC, which represents an aggressive histotype responsible for death in most of these patients [6]. MEN-2-associated MTC is bilateral and generally emerges from a multifocal/multicentric origin, and it is usually preceded by multifocal C-cell hyperplasia (CCH) [7]. *RET* is mutated in more than 95% of MEN-2 families. The sporadic cases are characterized by a unifocal clonal tumor cell population. Somatic *RET* mutations in the tumor tissue of sporadic MTC (S-MTC) have been reported at different rates, in the range 40-50%. Around 7-10% of apparently sporadic MTC are also found to have germline *RET* mutations [8, 9].

The natural history of MTC is variable and the main factors influencing survival are: the stage of the disease at the time of diagnosis, tumor size, lymph node involvement, age and sex of the patients, calcitonin doubling time and clinical variety of the MTC. Patients with MEN-2A show a better survival rate compared to patients with sporadic disease [10]. Patients with FMTC undergoing screening are younger and the disease is diagnosed at an earlier stage. Patients with MEN-2B have the highest risk of advanced-stage disease at presentation [10] and have a worse prognosis than do patients with MEN-2A and FMTC at a similar stage of the disease.

*Address correspondence to this author at the Endocrinology Unit, Catholic University, Largo Gemelli 8 - 00168, Rome, Italy;
Tel: +39063219418; Fax: +390632500063;
E-mail: corsello.sm@mclink.it

Table 1.　Classification of Multiple Endocrine Neoplasia Type 2

	MTC %	PHEO %	PHP %	Associated Diseases
MEN-2A	90	40-50	10-20	Hirschsprung's disease, cutaneous lichen amyloidosis
MEN-2B	100	50		Marfanoid habitus (midface hypergnathism, elongated long bones, scoliosis, slipped femoral head epiphysis, pectus and joint deformities), intestinal ganglioneuromatosis, mucosal neuromas
FMTC	95			Rare

Classification of MEN-2, including MEN-2A, MEN-2B and familiar medullary thyroid cancer (FMTC) with the occurrence of medullary thyroid cancer (MTC), pheocromocytoma (PHEO), primary hyperparathyroidism (PHP) and other diseases. From: reference [17].
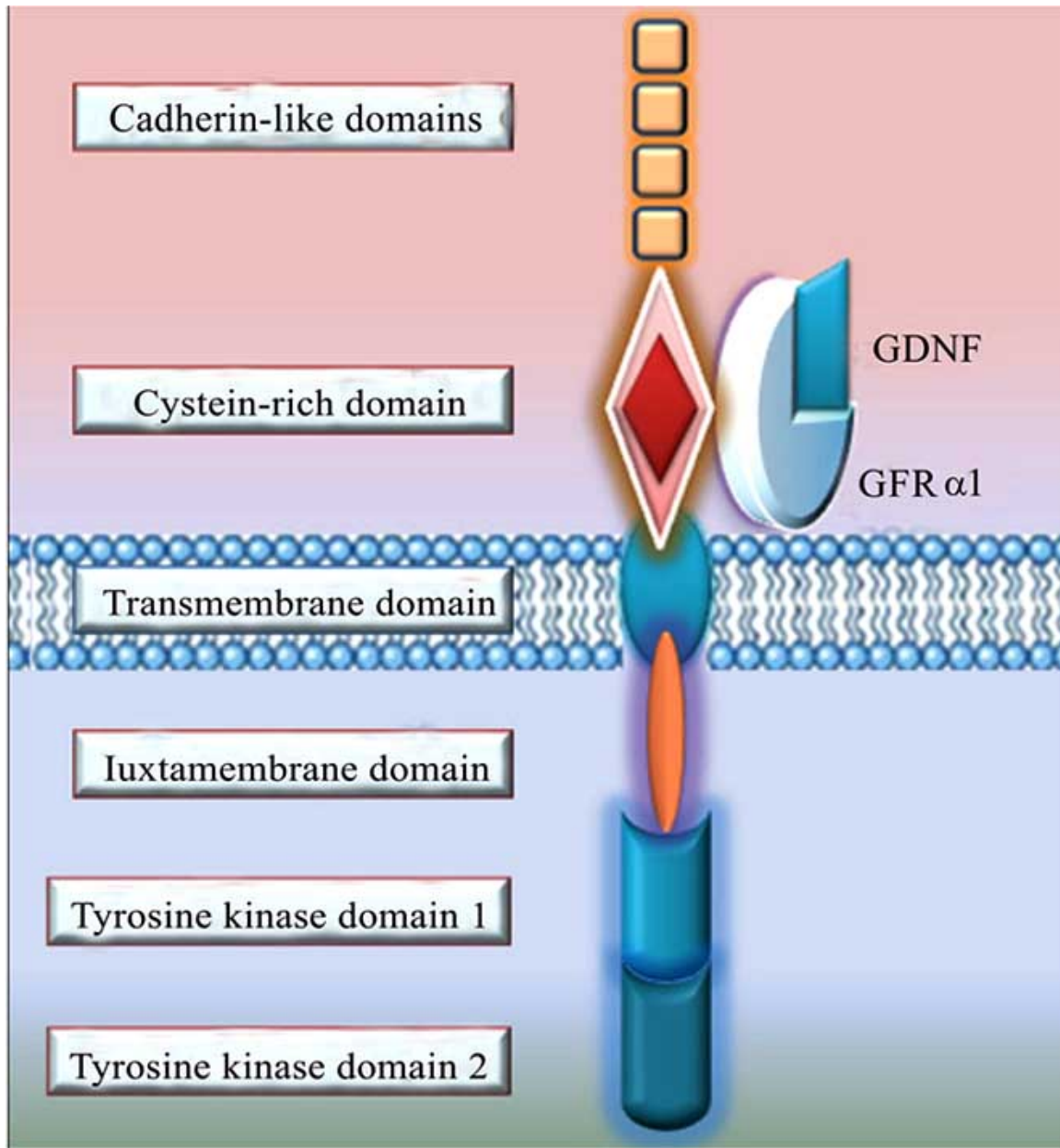

Fig. (1). Structure of RET.
RET is a single-pass transmembrane receptor belonging to the TK superfamily. In the extra-cellular domain, there are four cadherin-like domains and a juxtamembrane cysteine-rich region. In the intracellular region there are two tyrosine kinase subdomains that are involved in the activation of several intracellular signal transduction pathways. The glial cell-line-derived neurotrophic factor (GDNF) is a ligand of RET receptor: RET is activated by the interaction between ligand of the GDNF family and specific glycosyl-phosphatidylinositol-anchored coreceptors (GFRα1-4) [145].

PATHOPHYSIOLOGY OF RET

The *RET* proto-oncogene is located on chromosome sub-band 10q11.2 and comprises 21 exons. *RET* is mainly expressed in neuronal crest-derived and urogenital progenitor cells. It is required for the development of the enteric nervous system, kidney morphogenesis and differentiation of spermatogonia [9, 10]. *RET* encodes a typical single pass receptor tyrosine kinase (RTK) constituted by an extra-cellular, a transmembrane and a cytoplasmic domain [11] (Fig. **1**).

Activation of the RET receptor normally requires the construction of a complex including RET and coreceptors of glial cell line-derived neurotrophic factor (GDNF) (GFR1-4) [12]. This represents the functional multimolecular substrate for ligands of the GDNF family (GDNF, neurturin, persephin and artemin) which induce RET dimerization and mutual trans-autophosphorylation activity. Several tyrosine residues in the intracellular part of the RET molecule become phosphorylated upon receptor activation and provide "docking sites" for a number of signaling and adaptor proteins. This activation, in turn, leads to the assembly of different signaling complexes, which activate various signal transduction pathways. Particularly, Tyr1062 seems involved in the majority of cellular functions

regulated by the RET receptor and in the transforming potential of RET oncoproteins as well [10]. Several RET binding proteins have been identified, such as Shc, ShcC, Grb2 and Grb7/10, PLCg, Enigma, IRS1/2, FRS2, DOK1/4/5/6, c-Src, SH2-Bb, PKCa, Shank3, and STAT3. The recruitment of such signaling and adaptor proteins eventually results in the activation of downstream pathways involving RAS/RAF/ERKs, PI3K/AKT, JNKs, p38, ERK5 and PLCg, which, in turn, leads to gene expression regulation and biological response processes such as proliferation, survival, differentiation, migration and chemotaxis [10, 11, 13]. Recently, a direct interaction between RET and β-catenin signaling has been described in 2A-RET and 2B-RET transfected cell lines and in human papillary thyroid carcinoma (PTC) cell lines [14, 15].

Different alterations of the RET proto-oncogene correspond to specific human diseases. It has been demonstrated that "loss-of-function" mutations have a causative role in the development of Hirschsprung's disease, a polygenic disorder characterized by congenital absence of parasympathetic innervation in the lower intestinal tract [13].

By contrast, "gain-of-function" mutations, leading to aberrant activation of *RET*, are specific oncogenic
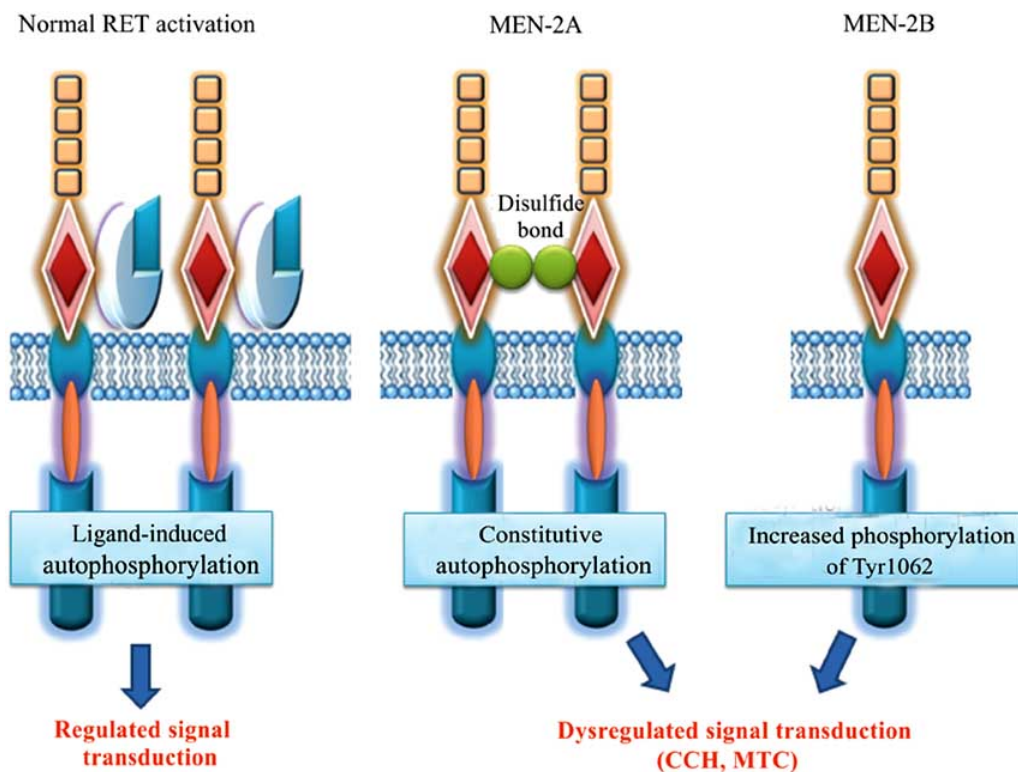

Fig. (2). Normal and oncogenic activation of RET.

In physiological condition, RET activation requires the binding of the GDNF ligand to glycosylphosphatidylinositol-anchored co-receptors (GFRα1). Ligand binding leads to RET dimerization, kinase activation and signaling to the nucleus. In MEN-2A mutations there is a ligand independent homodimerization through the formation of a covalent intermolecular disulfide bond in the cysteine-rich extra-cellular domain. As a consequence, dimerized RET mutants are constitutively activated without ligand stimulation. On the other hand, MEN-2B mutations activate the RET receptor in monomeric state, inducing increased phosphorylation of tyr1062 and altering the specificity of the thyrosine kinase domain.

From reference [1].

events in the thyroid gland contributing to the progression of inherited and non-inherited medullary or follicular thyroid cancer. Somatic gene rearrangements of *RET* have been found in PTC and S-MTC and germline point mutations in MEN-2A and 2B and FMTC.

In physiological conditions, RET kinase activation is dependent on ligand binding. Conversely, mutated RET in MTC and PTC cells shows a constitutive ligand-independent tyrosine kinase (TK) activity associated to transforming activity [10, 11, 13] (Fig. **2**). Germline dominant activating mutations in the extra-cellular or TK domains of RET have been identified as the initiating events in MEN-2 [10] (Fig. **2**). The mechanism of RET activation is different among the different syndrome subtypes.

MEN-2A

MEN-2A syndrome is the most common form of MEN-2 and is characterized by MTC (90%) in combination with pheochromocytoma (40-50%) and/or parathyroid hyperplasia or single adenoma (10-20%) in a single patient, or the presence of two or more tumor types in multiple members of a single family [16]. MTC is generally the first manifestation of MEN-2A and develops between the ages of 5 to 25 years [17]. A variant of MEN-2A associated with lichen amyloidosis has an earlier onset [18]. Mutations in *RET* codon 634, and to a lesser extent in *RET* codons 609, 611, 618 and 620 are related to a classic MEN-2 phenotype [19]. Mutations in *RET* codons 630, 790, 791, 804 and 891

are capable of producing the MEN-2A phenotype [19]. Mutations at codon 634 (exon 11) occur in 85% of cases with C634R being the most frequent RET alteration in MEN-2A [20]. Mutations at exon 10 account for a further 10–15% of cases, whereas mutations at other exons are rare (Fig. **3**; Table **2**).

Mutations identified in patients with MEN-2A affect one of six cysteine residues in the cysteine-rich region of the RET extra-cellular domain which is substituted by another amino acid. Cysteine residues are normally matched in intramolecular disulfide bonds and their loss by mutations may cause the formation of inter-molecular bonds through the partner cysteine residues, thereby causing ligand-independent homodimerization. This homodimerization results in the constitutive activation of the RET kinase, which, in turn, leads to permanent downstream signaling (Fig. **2**) [1, 21].

MEN-2B

MEN-2B syndrome is the less common, but the more aggressive form of MEN-2 (5-10% of cases). The onset of disease usually occurs during the first year of life. MEN-2B is characterized by MTC and pheochromocytoma, usually without hyperparathy-roidism. Other components of the syndrome are mucosal neuromas, intestinal ganglioneuromas, and marfanoid habitus with skeletal deformations and joint laxity, but without the vascular and ophthalmologic abnormalities that may be seen with other phakomatoses, such as von-Hippel Lindau syndrome. The skeletal abnormalities of MEN-2B may be due to
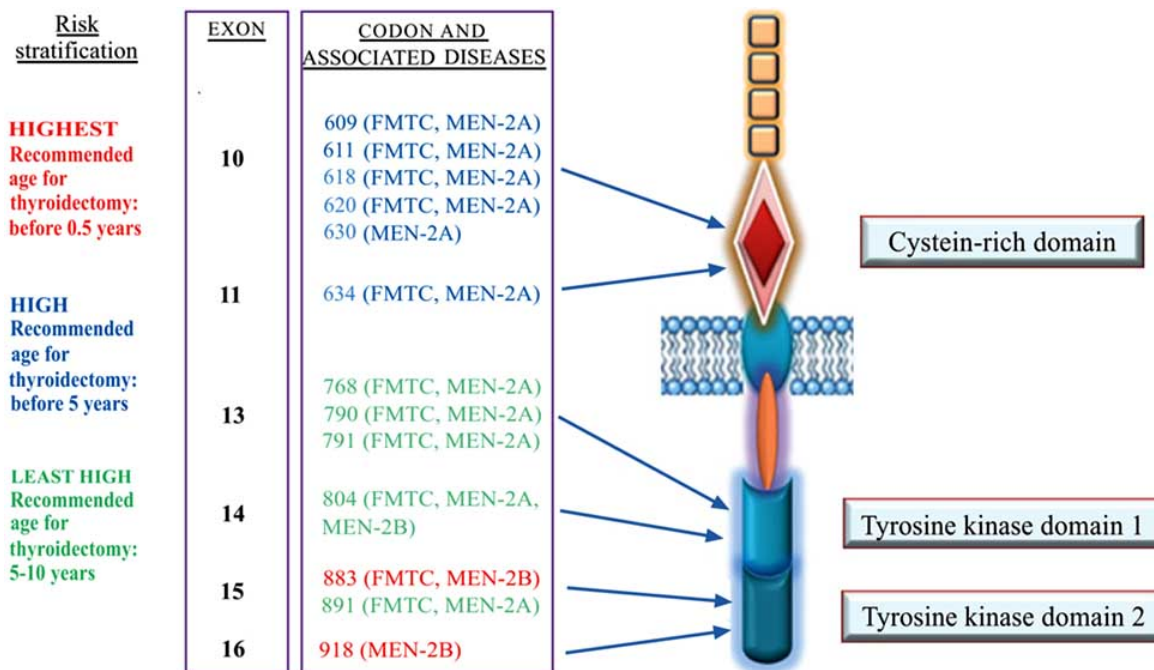

Fig. (3). RET codon mutations in MEN-2 syndromes and in FMTC. In round brackets associated hereditary MTC clinical varieties are indicated. On the basis of which RET codon is mutated, three levels of risk stratification to predict MTC development and aggressiveness are known. Highest risk mutations, suggesting preventive thyroidectomy before 6 months of age are written in red; for high risk mutation (blue color), total thyroidectomy is recommended before 5 years. Recommended age for thyroidectomy in least high mutations (green color) is 5-10 years.

Table 2.   RET Mutations Associated with RET Syndrome

Codon	Frequency	Risk Degree	Recommended Age for Surgery: Thyroidectomy Before Age (Years)
MEN-2A			
634	87	High	5
620	6	High	5
618	3	High	5
611	2	High	5
609	< 1	High	5
630		High	5
768		Least high	5-10
804		Least high	5-10
891		Least high	5-10
MEN-2B			
918	94	Highest	0.5
883	5	Highest	0.5
804	< 1	Least high	5-10
891		Least high	5-10
FMTC			
618	30	High	5
634	26	High	5
620	21	High	5
768	8	Least high	5-10
609	4	High	5
804	3	Least high	5-10
790		Least high	5-10
791		Least high	5-10
883		Highest	0.5
611		High	5

overexpression of chondromodulin, a regulator of cartilage and bone growth, which is reduced in this syndrome [19]. Patients affected by MEN-2B often lack a family history of MTC and harbor a *de novo* mutation [17].

In contrast with MEN-2A, mutations found in the RET gene of patients with MEN-2B primarily affect residues in the intracellular TK domain of the protein. These missense mutations result in the activation of the RET receptor in its monomeric state, thereby changing the conformational state of the TK domain catalytic core and its substrate specificity [13]. More than 95% of patients harbor a mutation at codon 918 (M918T, exon 16), whereas a few patients (<5%) may have a mutation at codon 883 (A833F, exon 15) (Fig. **3**). Moreover, increased autophosphorylation of tyr1062 has been described in patients with MEN-2B while MEN-2B cases associated with double germline mutations, have also been reported [22-24].

FAMILIAL MTC

FMTC is considered the mildest variant of MEN-2, with a clinical course more benign than MEN-2A and

MEN-2B. Patients have a strong predisposition to develop MTC, but a very low incidence of other manifestations of MEN-2A. Typically, FMTC patients have a late onset and their prognosis is relatively good. The diagnosis of FMTC can be made on the basis of at least four family members being affected. Yet a family history is often inadequate in establishing diagnosis of familial disease, and biochemical plus genetic screening often reveal FMTC in patients originally thought to have the sporadic form of the disease. Many of the mutations at extra-cellular cysteines responsible for MEN-2A are also found in FMTC (>80% of FMTC families). In patients with FMTC, the mutations identified in *RET* affect the cysteine-rich region of the extra-cellular domain as well as the TK domain, leading to low-level activation of the RET kinase. This aspect may explain the indolent penetrance phenotype of FMTC [10, 25]. Aggressive MTC tumors, though, have been reported in some cases harboring codon 804 mutations [26]. The International *RET* Mutation Consortium Analysis reported that only 10% of FMTC families showed a germline mutation in the intracellular domain of the *RET* [8], while the French Calcitonin Tumors Study Group found that a mutation located

within the intracellular domain of *RET* (at codons 768, 790, 791-exon 13, or codon 804-exon 14) is present in about half of FMTC families [27].

SPORADIC MTC

Approximately 80% of S-MTC have at least one subpopulation with the *RET* codon 918 mutation as a somatic mutation. In addition, other mutations at extra-cellular cysteines have been described [10, 13]. The functional significance of such somatic *RET* mutations in the pathogenesis of S-MTC and its role in cancer onset/progression is unclear [28]. It has been demonstrated that the presence of a somatic *RET* mutation correlates with a worse outcome of S-MTC patients in terms of presence of lymph node metastases at diagnosis, persistence of metastatic disease, and lower survival rate in a long-term follow up [29]. In these studies, however, patients with MTC were screened for only a few specific mutations, mainly in codon 918, without evaluating the correlation between the type of *RET* mutation and the patients' clinicopathological features.

This correlation was recently described by Moura *et al.* [30] who analyzed *RET* exons 5, 8, 10-16 in 51 S-MTCs, finding somatic mutations in 65% of tumors. Among the *RET*-positive cases, exon 16 was the most frequently affected (60.6%). Two novel somatic mutations in exon 11 were identified: a heterozygous point mutation at codon 630 (Cys630Gly) and a 18 bp deletion at nucleotide c.1881 associated with the same allele with a silent nucleotide substitution at codon 634 (Cys634Cys).

According to specific mutational patterns, a sub-classification for S-MTC consisting of three prognostic groups has been proposed. In Group 1, patients with mutations in *RET* exons 15 and 16 were included; in group 2, patients had other *RET* mutations; in group 3, patients did not have any *RET* mutations. Group 1 had higher prevalence (P=0.0051) and number of lymph node metastases (P=0.0017), and presented more often multifocal tumors (P=0.037) and persistent disease at last control (P=0.0242) than group 2. Detectable serum CTN levels at last screening and stage IV disease were significantly more frequent in group 1 than in the other groups. In particular, among the S-MTC, patients with *RET* mutations in exons 15 and 16 are associated with the worst prognosis. Tumors with other *RET* mutations have the most indolent course, and those with no RET mutations have an intermediate risk [30].

When pangenomic DNA microarrays were used to analyze the transcriptome of 4 familial and 9 S-MTC, the gene expression pattern allowed to distinguish 2 groups of sporadic tumors. The first group displayed an expression profile similar to that expressed by inherited *RET* 634 tumors. The second one presented an expression profile close to that displayed by inherited *RET* 918 tumors and included tumors from patients with distant metastases. In particular, genes involved in proliferation and invasion (PTN, ESM1, and CEACAM6) or matrix remodeling (COL1A1, COL1A2, FAP) were overexpressed [31].

Further studies, however, are needed to investigate the exact clinical role of genetic abnormalities recently described for S-MTC subtypes.

MOLECULAR PATHOGENESIS OF MTC

The pathogenesis of MTC is far from being thoroughly elucidated and there are probably several other genetic events in association with *RET* mutations that could be responsible for the different features of the disease. However, these additional mutations in both hereditary and S-MTC development are largely unknown. Recently, a co-causative role of other protooncogenes, such as *RAS* or *BRAF*, identified as having a role in thyroid carcinogenesis, has been proposed for MTC. Initially, gene sequencing analysis of *RAS* family members and *BRAF* did not reveal any mutation [32-35], excluding that *RAS/BRAF* gene mutations might exert a role in MTC development. Contrarily, a recent study on 17 patients affected by MTC showed that *KRAS* and *BRAF* are mutated in 41% and 68% of cases, respectively [36].

Similarly, loss-of-function mutations of tumor suppressor genes, such as retinoblastoma protein (pRb), tumor protein p53 and PTEN, or of cell cycle inhibitors (p27Kip1 and p18-INK4c) seem to predispose to MTC in rodents, but their role remains to be confirmed in humans [37]. Also loss of heterozygosity in the von-Hippel–Lindau (VHL) disease tumor-suppressor locus has been demonstrated to occur at the somatic level in FMTC [38].

For MTC, several studies suggested that the first genetic damage ("first hit") might not be silent, but may determine a growth stimulus on the target thyroid C-cells and/or adrenal chromaffin cells, such as in MEN-2. In fact, tumor development in these syndromes is probably preceded by a C-cell and/or adrenal chromaffin cell hyperplasia. The MTCs and pheochromocytomas originate as multifocal clonal tumors, which rise from a different cell clone. The stimulus for this polyclonal hyperplasia could result from the original chromosome 10 abnormality and other subsequent genetic events. Amplification of the mutant allele or loss of the wild-type *RET* allele may act as a 'second hit' in tumors of patients with MEN-2 [39]. These and other complex events might cause the progression of clonal carcinomas and involve chromosomal loci other than the germline defect on chromosome 10 [40].

Several signal transduction pathways have been identified as mediators of the oncogenic properties of mutated RET in MTC, including both the phosphatidylinositol 3-kinase (PI3K)/Akt3 and Raf-1/MEK/extra-cellular signal-regulated kinase (ERK) pathways (also referred to as Ras/mitogen-activated protein kinase/MAPK) [41]. The RET mutation upregulates PI3K/Akt signaling [42]. This pathway may

function by suppressing apoptosis, even if recent evidence suggests that regulation of cell cycle progression is a parallel mechanism resulting in prolonged cellular survival [41]. The PI3K/Akt signal transduction pathway has also been implicated in the control of CTN and chromogranin A (CgA) production [41]. Other signaling molecules have also been implicated in the development of MTC, such as Notch1/Hairy Enhancer of Split-1/Achaete-Scute complex like-16 and glycogen synthase kinase-3b [43, 44], IGFR, NTR, FGFR, HSP90 [37].

Maintenance of telomere length has been reported to be an absolute requirement for unlimited growth of human tumor cells and, in about 85% of cases, this is achieved by reactivation of telomerase, the enzyme that elongates telomeres. In the case of human MTC, it seems that telomerase activity (TA) is low, supporting the idea that this enzyme is not important for the tumorigenesis of MTC. This is in contrast to the data obtained in other carcinomas, where high TA has been correlated with malignant transformation. Indeed, abrogation of tumor suppressor protein function such as p53 and pRb have been described in MTC and could explain the low apoptotic rate of this tumor also in the absence of activating a telomere-stabilizing mechanism. On the other hand, it is possible that the low TA could also exert a protective role against cell death or stabilize the karyotype [45].

Van Veelen *et al.* [46] described the presence of somatic mutations in the cell cycle regulator P18 in human *RET*-associated MTCs, which cause an amino acid substitution in the cyclin dependent kinase-interacting region of P18 (INK4C). This inhibits P18 function and reduces its stability [46]. In fact, in human MTC, both hereditary and sporadic, frequently the loss of a specific part or the entire short arm of chromosome 1 is detected, with the most common break point on 1p32, where the tumor suppressor gene CDKN2C8p18 is located.

Dysregulation of metalloproteinase and their tissue inhibitor participate in the degeneration of the extra-cellular matrix and are associated with cancer progression. MMP-2 is one of the most active metalloproteinases in cancer development, and it has been suggested as a possible marker of the malignant phenotype and prognostic factor for MTC [47].

Another important consideration involves the correlation between type 2 deiodinase and MTC cells. The type 1 (D1) and type 2 (D2) deiodinases are responsible for catalyzing deiodination of T4 to T3 and, consequently, play a critical role in regulating intracellular T3 concentrations. D2 is expressed in normal and stimulated human thyroid tissue and has been evaluated as a possible marker of thyroid follicular cell differentiation. D2 is underexpressed in PTC [48], but increased in follicular thyroid carcinoma [49]. The role of iodine metabolism in MTC was demonstrated by Souza Meyer *et al.* [50], who showed that D2 is expressed in MTC tissues at levels comparable with normal human follicular thyroid cells.

The biochemical and molecular properties of D2 in the human MTC cell line TT seem to be preserved despite C-cell dedifferentiation. The presence of this D2 in TT suggests a possible role of thyroid hormones in human C-cell metabolism. Furthermore, the expression of the thyroid hormone receptor was demonstrated in all MTC samples and in human MTC cell lines, suggesting a potential role of locally produced T3 by D2 in this neoplastic tissue.

Angiogenesis

Angiogenesis is the process of forming new blood vessels from preexisting vasculature. Although vascular endothelium is usually quiescent in adults, active angiogenesis has been shown to be an important process for new vessel development and subsequent tumoral growth, progression, and spread. The angiogenic phenotype depends on the balance of proangiogenic growth factors such as vascular endothelial growth factor (VEGF) and inhibitors, as well as interactions with the extra-cellular matrix, allowing for endothelial migration. Endocrine glands are typically vascular organs and their blood supply is essential for normal function and tight control of hormone feedback loops. In addition to metabolic factors such as hypoxia, the process of angiogenesis is also regulated by hormonal changes such as increased estrogen, IGF-I and TSH levels. Thyroid tumors are more vascular than normal thyroid tissue. There is a clear correlation between increased VEGF expression and more aggressive MTC behavior and metastasis.

Overexpression of VEGF-A was correlated with stage, tumor size, and metastasis of MTC. MTC cells overexpress VEGF and RET inhibition has been correlated with VEGF downregulation [51, 52]. A direct correlation between the angiogenesis pathway and RET remains to be elucidated, although some hypotheses have surfaced. In fact, loss of heterozygosity in the von-Hippel–Lindau (VHL) disease tumor-suppressor locus occurs at the somatic level in FMTC [38]. RET and the protein encoded by the gene associated with VHL disease might act along the same pathway by controlling neuronal cell survival [53]. Additionally, loss of the VHL protein leads to increased expression of hypoxia-inducible transcription factor (HIF), thereby promoting expression of VEGF and tumor angiogenesis. Finally, the expression of VEGFR1 and VEGFR2 [54] has been detected in thyrocytes as well as in endothelial cells.

RET AS A THERAPEUTIC TARGET IN MTC

In recent years, the clinical validation of molecularly targeted drugs that inhibit the action of pathogenic oncoproteins, particularly TKs, essential for tumor onset and progression [55], represents one of the most exciting developments in cancer research. In MTC, a number of specific genetic alterations have been identified as tumor-initiating events [56-58]. Since the identification of the products of oncogenic forms of RET

as constitutively activated TKs [26], inhibition of RET enzyme activity has been viewed as an opportunity not only to investigate RET-dependent signaling and cellular functions, but also to explore the possibility of interfering with the RET oncogenic potential [59].

Biochemical and biological studies with RET inhibitors are usually performed on common cellular models of MTC, including RET oncogene-transformed murine fibroblasts or thyrocytes and human tumor cell lines expressing endogenous RET oncoproteins [59]. Only two MTC-cell lines are available, TT and MZCRC-1, harboring the MEN-2A-type C634W and the MEN-2B-type M918T RET mutation, respectively. A number of natural or synthetic compounds were reported to exhibit RET inhibitory activity. Among natural compounds, herbimycin A [60] and clavilactones [61] were first identified as RET inhibitors.

Several strategies aimed at blocking the activation and signaling of RET have been preclinically tested [9, 13]. They include the interference with formation of ligand-receptor complexes, dimerization, autophosphorylation, recruitment of adaptor proteins to various docking sites, and initiation of signal transduction cascades. Down-regulation leading to the disappearance of RET from the cell surface constitutes another important means of regulation and a potential target for therapy. Of particular interest, the elegant studies with dominant-negative RET mutants delivered by viral vectors which documented that the selective abrogation of oncogenic RET signaling in MTC cells results in loss of the neoplastic phenotype associated with apoptosis [1].

Additional strategies to block RET oncogenic activity have been reported, including soluble RET mutant ectodomain, gene ablation technologies such as ribozymes and silenting-RNA or molecules, neutralizing aptamers and monoclonal antibodies [1, 62, 63]. Despite the fact that these approaches are in early stages of development and are far from clinical application, they provided a solid preclinical background supporting the concept that RET oncogene and their products represent potential therapeutic targets [59].

Although almost all steps of RET and related pathways signaling cascade may be inhibited by relatively specific agents, the most advanced results have been obtained by competitive inhibition of RET activity by TK inhibitors (TKIs).

Like most of the known TKIs, synthetic RET inhibitors exhibit a "promiscuous" mechanism of action based on ATP competition. In fact, the available RET-TK inhibitors are not specific and were previously discovered as inhibitors of more than one TK, including RET-TK, with different IC50.

TARGETING RET WITH TKI: PRECLINICAL STUDIES AND CLINICAL TRIALS

Several classes of molecules have demonstrated inhibitory properties of RET kinase activity in preclinical

Table 3. Targeted Agents Under Preclinical Evaluation in Medullary Thyroid Cancer

Agents	Possible Mechanism(s) of Action	References
PP1, PP2	RET; Src kinases; Proteosomal degradation of RET	[65-68]
CEP-701, CEP-751	Trk family kinase; RET; NTR	[70, 71]
RPI-1	RET	[65, 72, 73]
NBL-1 (monoclonal antibody)	RET internalization	[87]
D4 aptamer	RET dimerization	[90]
Adenovirus-mediated gene therapy	RET gene	[91]
RET-selective ribozyme	Mutant RET mRNA and RET-mediated cell growth and transformation	[93]
RET- selective phosphatases	RET activity	[94]
Tipifarnib (R115777)	RAS-RAF-MAPK/ERK pathway (Ras farnesylation)	[104]
LY294002; KP372–1	PI3K/Akt pathway	[105, 118]
ADW742	IGF-1R	[109]
PD173074	FGFR-4	[108]
NVP-AEE788	VEGFRs and EGFR	[100, 101]
Vatalanib (PTK787/ZK222584)	VEGFRs	[102]
Cediranib (AZD2171)	VEGFRs	[103]
Pazopanib (GW-786034)	VEGFRs, PDGFR α/β, c-KIT	www.clinicaltrials.com

studies. They include the pyrazolo-pyridimidine inhibitors PP1 and PP2, the indolocarbazole derivatives CEP-701 and CEP-751, the 2-indolinone derivative RPI-1, and the anilinoquinazoline ZD6474 [64]. Despite an attractive mechanism of action and except for ZD6474 (vandetanib), these molecules are not yet available for clinical trials (Table **3**).

PP1 and PP2 inhibited enzymatic activity and transforming ability of NIH3T3 fibroblasts transfected with almost all types of RET/MEN-2A, RET/MEN-2B and RET/PTC1 and RET/PTC3 [65-68]. PP1 and PP2 also inhibit the Src family of kinases, a major downstream effector of RET-mediated mitogenesis. It remains unclear, therefore, whether the inhibitory effects of PP1 and PP2 on cell cultures arise from inhibition of RET [69]. PP1 induces RET/MEN-2A and RET/MEN-2B oncoprotein degradation *via* proteosomal pathways, providing an additional mechanism of RET inhibition [67].

The indolocarbazole derivatives CEP-701 and CEP-751 were originally developed to inhibit TRK family TK [70]. Additionally, these compounds demonstrated an inhibitory effect on RET autophosphorylation and proliferation of TT cells [70]. Moreover, CEP-751 inhibited TT cells growth in nude mice [70]. Interestingly, CEP-751 *in vivo* inhibitory effects increased when it was used in combination with irinotecan, a chemotherapeutic drug approved for metastatic colorectal cancer [71]. Irinotecan alone also has a strong effect on MTC xenograft growth in nude mice [71]. It remains to be determined, however, whether CEP-751 alone or in combination with irinotecan is effective in patients with MTC.

RPI-1 inhibited RET in human TT cells and RET/MEN-2A and RET/PTC1-expressing NIH3T3 cells [72]. RPI-1 decreased activation of RET downstream molecules including PLC, ERK, and AKT and reduced proliferation [73]. RPI-1 also showed antitumor effects in nude mice [65, 72, 73]. Interestingly, MTC cells showed only growth arrest rather than apoptosis induction after exposure to RPI-1 [73]. Nevertheless, inhibition of MTC tumor cell proliferation seems to be sufficient to induce its antitumoral effect.

Vandetanib is an anilinoquinazoline inhibitor of VEGF receptors (VEGFR; VEGFR2 and VEGFR3), epidermal growth factor receptor (EGFR) and RET phosphorylation and signaling [74, 75]. Vandetanib targets the enzymatic activity of both MEN-2 and PTC-related oncogenic RET and has an IC50 of 100 nmol. In addition, the compound inhibits tumor growth in RET/PTC transformed NIH3T3 cell xenografts [75]. It has been recently demonstrated that the compound docks into the ATP-binding pocket of RET [76]. Vandetanib inhibits the wild-type enzyme and most of the activated forms of RET, with the exception of RET molecules with mutations in residue Val[804] [76]. Mutation at this site has been proposed as a possible mechanism of acquired resistance to the drug [68]. In phase I clinical trials of patients with advanced non-small cell lung cancer (NSCLC) and other solid tumors,

including MTC, vandetanib was relatively well-tolerated after oral administration [77].

Wells *et al.*[1] reported the definitive results of an open-label phase II clinical trial of patients with metastatic hereditary MTC. Thirty patients with locally advanced or metastatic hereditary MTC and measurable disease (Response evaluation criteria in solid tumors, RECIST) were enrolled. The median duration of treatment was 172 days. Twenty percent of patients experienced a partial response, another 30% displayed stable disease, yielding a disease control rate of 50%. More than half of the patients had significant reductions in serum CTN levels. Overall, the drug was well tolerated.

A phase II trial of vandetanib for S-MTC patients with locally advanced and metastatic disease is currently underway. A randomized phase II trial comparing vandetanib to placebo in patients with hereditary or S-MTC is being planned.

Imatinib mesylate (STI-571) is a 2-phenylaminopyrimidine derivative acting as an inhibitor of the c-*KIT* and the receptor for PDGFR TKs. It received FDA approval for clinical use as single treatment of GIST and CML in 2004 [78]. Imatinib has been shown to display inhibitory activity against RET/MEN-2A and RET/MEN-2B in MTC-derived cell lines. Imatinib induces RET degradation through nonproteasomal pathways [79]. Furthermore, it inhibits RET TK, but at high concentrations. Despite this suboptimal pharmacological property, the drug was the first TKI used in two trials with 9 and 15 patients affected by MTC, respectively. Unfortunately, imatinib resulted in limited efficacy and did not induce any tumor response [80, 81].

Sunitinib malate (SU 11248) is an indolinone derivative approved for RCC and refractory GIST. It is a multikinase inhibitor, highly active against RET and is under investigation in phase II trials on patients affected by unresectable differentiated thyroid cancer (DTC), refractory to ^{131}I and advanced MTC (www.clinicaltrial.gov; NCT00381641).

Preliminary results of 16 patients (3 with MTC) are available. Seven patients (44%), all with DTC, had a FDG-PET response. Response rates at 3 months, according to RECIST criteria, were not reported. No grade 4 toxicities have been registered. The main grade 3 toxicities included neutropenia (28%), leukopenia (17%), fatigue (11%), hand-foot syndrome (11%), and gastrointestinal bleeding (11%). Grade 3 diarrhea, mucositis or atrial fibrillation were reported in 6% of patients[2].

Sorafenib tosylate (BAY 43-9006) was initially developed to target the Raf family of kinases, mainly B-Raf and C-Raf. It also inhibits other kinases including VEGFR2, platelet-derived growth factor receptor

---

[1]Wells SA, Gosnell JE, Gagel RF, Moley JF, *et al.* Abstract No. 6018, ASCO Annual Meeting, 2007.
[2]Goulart B, Carr L, Martins RG, Eaton K, Kell E, Wallace S, Capell P, Mankoff D. Abstract No. 6062, ASCO Annual Meeting, 2008.

(PDGFR), c-Kit, FLT3 and RET. The drug has recently been approved as treatment of advanced RCC and hepatocarcinoma. Carlomagno *et al.* showed that 20 to 50 nmol/L sorafenib inhibits 50% (IC50) of NIH 3T3 fibroblasts expressing one of three oncogenic versions of RET (RET/PTC3, RET/C634R, or RET/M918T) and showed almost complete inhibition with 100 nmol/L. Cells expressing both RET/V804L and RET/V804M, resistant to vandetanib, were sensitive to sorafenib at 110 and 147 nmol/L, respectively [82]. Very preliminary results reported that sorafenib mesilate obtained objective responses in two out of five patients with metastatic MTC[3]. Stable disease was registered in the other three patients after six months of treatment. Interestingly, after 2-3 months CTN levels were decreased by more than 50% from baseline in all patients.

Sorafenib is under investigation in two phase II trials recruiting patients that are affected by metastatic or unresectable thyroid cancers, including MTC (www. clinicaltrials.gov; NCT00601783; NCT00654238).

Motesanib diphosphate (AMG 706), is a multikinase inhibitor of VEGFRs, PDGFR, c-kit [83]. It also inhibits wild-type but not mutant RET *in vitro* and induces regression of MTC xenografts in mice[4]. In a phase II study 91 patients with locally advanced or metastatic, progressive or symptomatic MTC received motesanib 125 mg/d orally for up to 48 weeks or until unacceptable toxicity or disease progression. Only two patients (2%) achieved objective response (95% CI,0.3% to 7.7%); their duration of response was 32 weeks (censored) and 21 weeks (disease progressed). Eighty-one percent of patients had stable disease (48% had durable stable disease ≥ 24 weeks), 8% had disease progression as best response, and 9% were not evaluated. Median progression-free survival was 48 weeks (95% CI, 43 to 56 weeks). Among patients with tumor marker analysis, 69 (83%) out of 83 and 63 (75%) out of 84 had decreased serum CTN and carcinoembryonic antigen during treatment, respectively, compared with baseline. The most common treatment-related adverse events were diarrhea (41%), fatigue (41%), hypothyroidism (29%), hypertension (27%), and anorexia (27%) [84]. In pharmacokinetic analyses, motesanib trough concentrations were lower compared with DTC patients from a parallel cohort study [85].

XL184 is a potent, orally available small molecule inhibitor of MET and VEGFR2/KDR and also inhibits KIT, RET, FLT3, and Tie-2. Salgia *et al.* [86] reported a phase I study on 25 patients with advanced malignancies, including three patients with MTC. Using dose escalation and assessing response by RECIST criteria, as well as serologic measurements of CTN, VEGF-A, soluble VEGFR2 (sVEGFR2), and Ang2, after a median follow up of over 12 months, the patients

affected by MTC (one of whom had a documented RET mutation) had substantial reductions in plasma CTN and stable disease for over 6 months, without serious toxicities. A phase III trial is ongoing to evaluate the progression-free survival (PFS) with XL184 in comparison with placebo in subjects with unresectable, locally advanced, or metastatic MTC.

OTHER STRATEGIES TO INHIBIT RET

The first step in the activation of RET is the binding of the growth factor (GF) – ligand to the complex GF-receptor/co-receptor. Therefore, targeting GFs, or the binding site of the receptor/coreceptor, represents a straightforward approach for RET inhibition. Unfortunately, monoclonal antibodies against the RET ligand or receptor/co-receptors have not yet been synthesized. Yano *et al.* [87] generated in a neuroblastoma model an antibody capable of inducing internalization of RET, but its activity remains unknown.

The second step in RET activation is dimerization. It has been demonstrated that the introduction in the membrane of small peptides corresponding to the transmembrane domain could compete with dimerization and thus inhibit the kinase activity of some human cancer cells (i.e., those overexpressing ErbB2 and EGFR) [88].

Aptamers are single-stranded DNA or RNA oligonucleotides that have specific three-dimensional structures and bind to target molecules. Aptamers are usually created by selecting them from a large random sequence pool, but natural aptamers also exist in riboswitches [89]. Several unique properties of aptamers, including high binding specificity, low immunogenicity, structural stability and ease of synthesis, have made aptamers promising agents for directed therapy against cancer targets [89]. Aptamers with anti-neoplastic activity against extra-cellular, cell membrane and intracellular targets have been developed [89]. An aptamer that targets activated RET and blocks RET-dependent intracellular signaling by interfering with receptor dimerization has recently been described [90]. The neutralizing nuclease-resistant D4 aptamer was capable of binding and inhibiting wild-type RET and RET/MEN-2A on the cell surface inhibiting the constitutive RET dimerization [90]. It is noterworthy that only MEN2A mutated MTCs would benefit from inhibiting RET dimerization, as the M918T mutation present in MEN2B-MTC and most cases of sporadic MTC, results in activation of monomeric RET (Fig. **2**). However, the efficacy of the D4 aptamer against RET-associated tumors remains to be established.

Another new treatment approach of MTC is the integration of new genetic material into the genome – "gene therapy". This approach can be used to replace defective genes or block the effects of unwanted ones by the introduction of a counteracting gene. Drosten *et al.* [91] reported that adenovirus-mediated transduction of dominant negative RET into TT cells reduced expression of oncogenic RET receptors on the cell

[3]Kober F, Hermann M, Handler A, Krotla G. Abstract No. 14065, ASCO Annual Meeting, 2007.
[4]Coxon A, Bready JV, Hughes P. *et al.* Proc Am Assoc Cancer Res 2007; 48: 71 (abstr LB-283).

surface. They also reported that inoculation of dominant negative RET-expressing MTC cells into nude mice led to an almost complete suppression of tumor growth [91, 92]. These results suggest that inhibition of oncogenic RET expression by a dominant-negative RET mutant is an effective approach for MTC treatment, although many issues remain to be resolved before viral vectors can be used *in vivo*.

Another gene therapy approach might be the introduction of a RET-selective ribozyme that specifically cuts mutant RET mRNA and blocks RET-mediated cell growth and transformation [93]. The ectopic expression of RET- selective phosphatases has also been shown to efficiently block RET activity [94].

ANGIOGENESIS AS A THERAPEUTIC TARGET

Several findings suggest that antiangiogenic therapy of patients with MTC is rational. As previously reported, all the inhibitors of the RET kinase activity that are currently under clinical evaluation have activity against the TK of VEGFRs. These compounds might, therefore, have a combined effect inhibiting RET in tumor cells and VEGFRs in endothelial cells. To date, VEGF targeting is a widely used anti-cancer therapeutic approach. VEGF-targeted therapy acts through various mechanisms: inhibition of new vessel formation, apoptosis of pre-existing vessels, blockade of endothelial cell progenitors, and vessel constriction (with reduced blood flow and ischemia) [95]. Bevacizumab, a humanized monoclonal antibody against VEGF, is the first antiangiogenic agent approved for the treatment of patients affected by metastatic colorectal cancer, breast cancer, and NSCLC, in combination with standard chemotherapy [96] or in combination with interferon in metastatic renal cancer [97]. Bevacizumab inhibited the growth of mouse xenografts of thyroid cancer cells [98]. However, clinical trials exploring the activity of bevacizumab in MTC patients have not been conducted.

Several multi-targeting TKIs that block VEGFRs have shown promising clinical activity against several solid tumors, including MTC. Axitinib (AG-013736), a VEGFR inhibitor with no known anti-RET activity, has been tested in a phase II trial that involved 60 patients with thyroid cancer derived from either medullary or follicular cells. Eleven patients (16%) with MTC were involved: partial responses were observed in 2 MTC patients and stable disease lasting more than 16 weeks was reported in another 3 MTC patients [99]. Preclinical studies with NVP-AEE788, a dual VEGFR and EGFR inhibitor, in DTC cell lines [100, 101] and with vatalanib (PTK787/ZK222584) [102], a pan-VEGFR TKI, in thyroid carcinoma mouse xenografts, showed promising results. Cediranib (AZD2171), another pan-VEGFR TKI, demonstrated inhibitory activity on tumor growth and prolonged animal survival in an orthotopic nude mouse model of anaplastic thyroid cancer [103]. In addition, pazopanib (GW-786034), another multi-targeted VEGFRs inhibitor, is

undergoing clinical evaluation in thyroid cancer patients.

TARGETING OTHER KINASES

Although the inhibition of the RET pathway is actually one of the most studied for therapeutic purposes, other signal transduction pathways have been implicated as contributing to the growth and functional activity of MTC and are considered promising as therapeutic targets (Table **3**). In particular, inhibition of RAS–RAF–ERK [104], PI3K–AKT [105], NFκB [106] and the glycogen synthase kinase-3 (GSK-3) [107] pathways have a promising effect in cultured MTC cells. Additionally, data from preclinical experiments have shown that the inhibition of fibroblast growth factor (FGF) receptor 4 (FGFR4) [108] and the insulin-like growth factor I receptor (IGF-1R) [109] reduces the growth of MTC cells.

RAS-RAF-MAPK-ERK PATHWAY

Tipifarnib (R115777) is a member of a novel class of agents developed to inhibit the farnesylation of Ras and other proteins. Tipifarnib shows antiproliferative effects against many human tumor cell lines. Tipifarnib has not shown direct activity against RET kinase, but it can inhibit RET signaling through the MAPK pathway. This compound was active in myelodysplastic syndrome and in acute myelogenous leukemia [110, 111]. In a patient affected with S-MTC, with a novel somatic deletion in exon 11 of the RET gene, a rapid and marked response to a regimen incorporating both sorafenib and tipifarnib was observed. It has been hypothesized that tipifarnib, which can effect downstream signaling of RET kinase, may synergistically act with sorafenib [112].

The activation of the Raf-1 pathway in MTC cells leads to significant growth suppression [113] and is associated with a reduction in CTN and CgA [107]. Moreover, it has been shown that growth inhibition by Raf-1 activation in the MTC-TT cell line induces an autocrine–paracrine protein, leukemia inhibitory factor, and this alone could mediate differentiation and cell growth inhibition [113]. Activation of the Raf-1 pathway in these cells also led to inactivation of GSK-3β by phosphorylation at Ser-9 [114], indicating a possible crosstalk with other pathways involved in growth regulation.

GSK-3 AS A POTENTIAL TARGET FOR MTC GROWTH REGULATION

GSK-3 is a serine/threonine protein kinase that was first described as playing a role in the regulation of glycogen synthesis [115]. GSK-3β, an isoform of GSK-3, is involved in many cellular processes, including metabolism, embryonic development and cell differentiation, proliferation, and survival [44]. GSK-3α has been shown to be involved in the regulation of

cellular proliferation [116, 117]. In contrast to other kinases, GSK-3β becomes inactivated by phosphorylation in response to signaling cascades [117]. GSK-3β regulates other molecules such as β-catenin, MAPK kinase 1, ERK-1/2, c-Myc, c-Jun, murine double minute 2, Mcl-1, and heat shock factor by phosphorylation, and therefore modulates diverse intracellular signaling pathways that are known to play key roles in cancer biology.

Kunnimalaiyaan *et al.* [44] have recently shown that inactivation of GSK-3β with lithium chloride resulted in MTC differentiation and cell growth inhibition. Based on these studies, a clinical trial using lithium as treatment of patients with metastatic MTC was initiated.

PI3K–AKT PATHWAY

PI3K–Akt is one of the major downstream pathways of RET. Its activation has a central role in thyroid tumorigenesis [118], but little is known about its role in regulating the growth of MTC tumors. The exposure of MTC cells to LY294002, a PI3K inhibitor, resulted in a dose-dependent reduction in cellular proliferation and neuroendocrine tumor markers [105]. The reduction in growth is mediated by apoptosis [105]. In addition, KP372–1, an Akt inhibitor, has been shown to inhibit cell proliferation and induce apoptosis in thyroid cancer cells [118].

NERVE GROWTH FACTOR RECEPTOR PATHWAY

The neurotrophin receptor (NTR) family includes 3 receptors with TK activity (NTR1, NTR2 and NTR3). Changes in their expression are involved in thyroid C-cell transformation [119, 120]. Particularly, NTR2 activity is reduced and NTR3 is up-regulated in MTC [121] CEP-751, demonstrating inhibition of RET and NTR TK, and showing a cytostatic effect in MTC cells [71].

INSULIN-LIKE GROWTH FACTOR RECEPTOR PATHWAY

IGF-1R is a ubiquitous transmembrane TK, structurally similar to the insulin receptor (IR). The α-subunit of IGF-1R binds IGF-I, IGF-II, and insulin at supraphysiological doses; the β-subunits contain the TK domain [122]. IGF-II and IGF-1R are over-expressed in many cancer types, including MTC [123]. IGF-1R up-regulation was found to mediate resistance to TKIs in different types of cancer cells. IGF-I and IGF-1R are over-expressed in thyroid cancer, particularly in the most aggressive variants [124]. Importantly, IGF-I and insulin are essential for the mitogenic action of TSH and EGF in thyroid follicular cells [123]. ADW742 is a specific inhibitor of IGF-1R phosphorylation and of its signaling pathway. This drug is cytotoxic for follicular and MTC-derived cancer cells [109].

FIBROBLAST GROWTH FACTOR RECEPTOR PATHWAY

FGF receptors (FGFRs) comprise a subfamily of RTKs that are master regulators of a broad spectrum of cellular processes, including apoptosis, proliferation, migration, and angiogenesis. Deregulation of FGFR signaling by activating mutations or ligand/receptor overexpression could allow these receptors to become constitutively active, leading to hematopoietic and solid tumors [125]. Four FGFRs with TK activity have been described (FGFR-1, -2, -3 and -4) [126]. Mutations or genetic alterations involving FGFRs have not been identified in thyroid cancer [126]. Two ligands of FGF (FGF1 and FGF2), the basic FGF, a potent angiogenic factor, is increased in differentiated and anaplastic thyroid cancer and MTC [127-129]. Increased expression of FGFR-1, -3 and -4 has been observed also in malignant thyroid tumors [130]. FGFR-2 expression, instead, was downregulated in thyroid cancer [131]. FGFR-4 is mostly expressed in aggressive thyroid tumor types and MTC cells [132].

PD173074 is a FGFR-4-TK competitive inhibitor. The drug produced the abrogation of FGF-1-mediated FGFR-4 phosphorylation in TT cells and significant inhibition of cell proliferation and tumor growth *in vivo*. Moreover, the combination of STI571 and PD173074 resulted in greater suppression of cell proliferation *in vitro* and tumor control *in vivo* than that achieved with either agent alone. These data highlight RET and FGFR-4 as therapeutic targets and suggest a potential role for the combined use of TKIs in the management of inoperable MTC [108].

Other TKIs, such as sorafenib, sunitinib and pazopanib, under clinical evaluation in thyroid cancer patients, exert anti-FGFR activity as well [132].

PROTEASOME INHIBITORS

The 26S proteasome is a large ATP-dependent multimeric complex that degrades intracellular proteins that have been targeted for proteolysis by the process of ubiquitination [133]. Several key regulators of transcription and growth/apoptosis, such as nuclear factor-κB (NF-κB) inhibitor (IκB), p53, c-*myc*, and c-Jun N-terminal kinase (JNK), are known substrates for proteasomal degradation [134]. NF-κB was implicated in the pathophysiology of both anaplastic [135] and medullary [136] carcinomas, suggesting that novel therapies targeting NF-κB may be effective in these malignancies.

Proteasome inhibitors constitute a novel class of antitumor agents with preclinical evidence of activity against hematological malignancies and solid tumors [136]. Specifically bortezomib, a boronic acid dipeptide proteasome inhibitor, is approved by the U.S. FDA for use in relapsed refractory multiple myeloma [136] and is currently being evaluated in a variety of other hematological and solid malignancies [137].

Mitsiades *et al.* [138] investigated the effect of bortezomib in a panel of medullary and anaplastic carcinoma cells *in vitro* and defined apoptotic pathways triggered by this novel anticancer agent. Bortezomib-induced apoptosis is mediated by caspases and may be modulated by the mitochondria and the Bcl-2 family members. The bortezomib-sensitivity of TT cells was reduced in the presence of IGF-I, suggesting that the antitumor activity of bortezomib might be enhanced by inhibition of IGF-I and its downstream signaling. In addition, the combination of bortezomib with conventional chemotherapeutic agents produced a synergistic effect.

However, despite *in vitro* studies having shown bortezomib to be active against a broad range of thyroid cancer cell lines, including MTC, the specific role of proteasome inhibitors as useful targeted therapy of MTC remains to be explored in clinical trials. Given the activity of bortezomib and the role of the proteasome in regulating several cellular pathways, a phase I/II study combining bortezomib with vandetanib as treatment for patients affected by advanced solid tumors, with a focus on patients with MTC, has been started (NCT00923247; www.clinicaltrial.gov).

THE NOTCH-1–HES-1–ASCL-1 SIGNALING PATHWAY

Notch-1 is a multifunctional protein that regulates cellular differentiation, development, proliferation, and survival in a variety of contexts. Its signaling pathway controls cell fate in multiple developmental programs and its dysregulation has been implicated in the oncogenesis of several types of cancer.

Notch-1 signaling might have a tumor suppressor role of in MTC tumors and cell lines. The activation of Notch-1 significantly reduced the growth of MTC-TT cells [43]. Furthermore, Notch-1 regulated CTN levels in a dose-dependent manner and the levels of reduction in growth and hormone production depended on the amount of Notch-1 protein present in the cell [43]. A lack of active Notch-1 protein was found in tumor tissues and MTC cell lines, whereas neuroendocrine markers, such as CgA and ASCL-1, were highly expressed. Activation of doxycycline-inducible Notch-1 in TT cells by varying the concentration of doxycycline led to a dose-dependent increase in Notch-1 protein and Hairy and Enhancer of Split homolog-1 (HES-1) protein. As expected, the level of Achaete-Scute Complex-Like homolog 1 was reduced with an increase in Notch-1 [43]. Moreover, the levels of reduction in growth and hormone production depended on the amount of Notch-1 protein present in the cell [43]. These observations suggest that activation of Notch-1 signaling may be a potential target to treat patients with MTC tumors.

Interestingly, Greenblatt *et al.* examined the effects of valproic acid, a histone deacetylase (HDAC) inhibitor, on cell proliferation in human MTC and particularly on Notch 1 expression. Notch 1 is absent in MTC cells at baseline, but valproic acid treatment leads to an activation of Notch1 protein that, in turn, induces cell apoptosis inhibiting tumor growth [139]. However, the exact mechanism by which valproic acid activates Notch1 signaling is yet to be determined. The induction of the Notch-1 signaling cascade is also related to the anti-proliferative and pro-apoptotic effect of the suberoyl bis-hydroxamic acid (SBHA), a relatively new HDAC inhibitor [140]. Nude mice injected with human MTC and then treated with SBHA demonstrated a mean 55% inhibition of tumor growth [140]. In particular, SBHA caused an increase in p21(CIP1/WAF1) – a p53 inducible protein, p27(KIP1) – a cyclin-dependent kinase inhibitor, cleaved caspase-9, cleaved caspase-3, and cleaved polyADP-ribose polymerase, with a concomitant decrease in cyclin D1 and cyclin B1 [140]. This indicates that the demonstrable growth inhibition was due to both cell cycle arrest and apoptosis. Moreover, SBHA downregulated the cell survival proteins Bcl-2 and Bcl-X(L), but upregulated the apoptotic proteins Bax, Bad, and Bmf [141]. These findings suggest that Notch-1 activation with HDAC inhibitors may present a promising new form of targeted biological therapy for the treatment of patients with MTC.

HEAT SHOCK PROTEINS

Many oncogenic protein kinases depend on the molecular chaperone heat shock protein 90 (HSP90) for correct maturation and activity. 17-allylamino-17-demethoxygeldanamycin (17-AAG) is an antibiotic of the ansamycin family with specific inhibitory activity against HSP90. 17-AAG resulted in the loss of oncogenic RET activity and effective TT cell-growth inhibition, suggesting a requirement for HSP90 action by activated RET [142]. It remains to be determined, however, whether RET oncoproteins are real targets of HSP90 [1]. A phase II trial is ongoing to determine the 1-year treatment failure rate in patients with inoperable, advanced or metastatic DTC or MTC, treated with 17-AAG (www.clinicaltrials.gov; NCT00118248). Although preclinical results seem encouraging, the clinical development of these agents, in terms of long-term toxicity profiles, clinical benefit, and the development of drug resistance, is the subject of intense research.

CONCLUSIONS AND CHALLENGES

At present, surgery represents the only curative treatment of MTC. Unfortunately, about two-thirds of patients experience relapse and die of the disease. Although the majority of patients survive for decades, there is still a need for novel therapeutic approaches to improve outcomes in terms of clinical response and quality of life.

A growing understanding of molecular pathogenesis of cancer has allowed the development of multiple target agents in MTC. Some novel compounds, mainly TKI of RET and angiogenesis inhibitors, presently under evaluation in clinical trials, have clearly shown

that they are effective with a low toxicity. Nevertheless, the activity and toxicity profile of the majority of other promising targeted agents requires further testing in clinical trials.

We are at the beginning of the clinical evaluation of a new therapeutic approach, but researchers are aware that several hurdles remain to be overcome in the clinical evaluation of targeted agents. They include the recognition in preclinical studies of appropriate cell systems and animal models to define and characterize the best target(s) and the real activity of targeted compounds of interest. Regarding *in vitro* testing, if RET is the target of interest, putative therapeutic compounds should be tested preclinically for their antitumor effect on cells expressing RET molecules with different mutations as well as on cells expressing wild-type RET. *In vivo*, a number of mouse models bearing mutations of *RET* and other genes, which spontaneously develop MTC are now available and would provide a better preclinical model than xenografts.

Another problem is the selection and validation of surrogate markers for target inhibition combinations of agents against different targets (e.g., expression/phosphorylation of receptor of interest in pre- and post-treatment tumor biopsies, although this approach appears ethically questionable). In parallel, the selection of patients most suitable to receive the agent(s) of interest is a major challenge. To this end, genotyping patients enrolled in clinical trials, for example in order to identify a mutation in the targeted kinase, such as RET, or a genome-wide profiling, is a promising approach [143]. Potential relationships between toxicity and drug efficacy should also be evaluated by pharmacokinetic/genomic studies to ensure that adequate plasma drug concentrations are achieved. Such studies are particularly relevant in patients with MTC because diarrhea can induce drug malabsorption [144].

Among the most important methodological issues in MTC clinical trial is the evaluation of response. In fact, the response rate according to RECIST guidelines does not necessarily predict a benefit in terms of progression-free survival or overall survival in MTC [SCH 144]. Overall survival cannot be used to assess response in patients with slowly progressing diseases. Hence, the time to progression might be a better end-point to measure the durability of treatment response, the value of stable disease, and the overall efficacy of treatment, especially if crossover from the placebo to the treatment arm is allowed. Further end-points, such as progression free survival, have been proposed but need to be validated as predictive measures of efficacy.

Another essential observation, which constitutes a major problem, is that the relevance of clinical trials depends on the power of their statistical design, which is related to the number of patients accrued. Unfortunately, most of the mentioned clinical trials enrolled small cohorts of patients. Furthermore, the

MTC population includes different subtypes of disease with specific mutations and therapeutic compounds might have variable efficacy on a particular subtype of disease. Consequently, future clinical studies should be balanced according to this aspect. To these aims, since MTC is a rare cancer, a major effort needs to be made by endocrinologists and oncologists to participate in multi-institutional studies in order to improve accrual of patients in well designed clinical trials. Finally, clinical trials should incorporate potentially prognostic and predictive biomarkers which may allow for more accurate identification of patients who will benefit from therapies under evaluation.

A rational and coordinated approach including biomarker studies throughout the drug development process appears to be the best way to define the oncogenic mechanisms driving the progression of MTC, as well as the real efficacy of novel therapeutic strategies that can improve therapeutic options offered to patients affected by MTC.

ABBREVIATIONS

CCH	=	C-cell hyperplasia
CgA	=	chromogranin A
CTN	=	calcitonin
D1	=	type 1 deiodinase
D2	=	type 2 deiodinase
DTC	=	differentiated thyroid cancer
FGF	=	Fibroblast growth factor
FGFR	=	fibroblast growth factor receptors
FMTC	=	familial MTC
GDNF	=	glial cell line-derived neurotrophic factor
GF	=	growth factor
GSK-3	=	glycogen synthase kinase-3
HDAC	=	histone deacetylase
HES-1	=	Hairy and Enhancer of Split homolog-1
HSP90	=	heat shock protein 90
IGF-1R	=	insulin-like growth factor I receptor
IR	=	insulin receptor
JNK	=	Jun N-terminal kinase
MEN-2	=	type 2 multiple endocrine neoplasia
MTC	=	medullary thyroid carcinoma
NF-κB	=	nuclear factor-κB
NTR	=	neurotrophin receptor
PTC	=	papillary thyroid carcinoma
RTK	=	receptor tyrosine kinase
SBHA	=	suberoyl bis-hydroxamic acid
S-MTC	=	sporadic MTC

TA = telomerase activity

TK = tyrosine kinase

TKI = tyrosine kinase inhibitors

VEGF = vascular endothelial growth factor

VHL = von-Hippel–Lindau

REFERENCES

[1] Drosten M, Pützer BM. Mechanisms of Disease: cancer targeting and the impact of oncogenic RET for medullary thyroid carcinoma therapy. Nat Clin Pract Oncol 2006; 3: 564-74.

[2] Leboulleux S, Baudin E, Travagli JP, Schlumberger M. Medullary thyroid carcinoma. Clin Endocrinol 2004; 61: 299-310.

[3] Gimm O. Thyroid cancer. Cancer Lett 2001; 163: 143-56.

[4] Hundahl SA, Fleming ID, Fremgen AM, Menck HR. A National Cancer Data Base report on 53,856 cases of thyroid carcinoma treated in the U.S., 1985-1995. Cancer 1998; 83: 2638-48.

[5] Hubner RA, Houlston RS. Molecular advances in medullary thyroid cancer diagnostics. Clin Chim Acta 2006; 370: 2-8.

[6] Boikos SA, Stratakis CA. Molecular mechanisms of medullary thyroid carcinoma: current approaches in diagnosis and treatment. Histol Histopathol 2008; 23: 109-16.

[7] Block MA, Jackson CE, Greenawald KA, Yott JB, Tashjian AH Jr. Clinical characteristics distinguishing hereditary from sporadic medullary thyroid carcinoma. Treatment implications. Arch Surg 1980; 115: 142-8.

[8] Eng C, Clayton D, Schuffenecker I, et al. The relationship between specific RET proto-oncogene mutations and disease phenotype in multiple endocrine neoplasia type 2. International RET mutation consortium analysis. JAMA, 1996; 276: 1575-9.

[9] De Groot JW, Links TP, Plukker JT, Lips CJ, Hofstra RM. RET as a diagnostic and therapeutic target in sporadic and hereditary endocrine tumors. Endocr Rev 2006; 27: 535-60.

[10] Arighi E, Borrello MG, Sariola H. RET tyrosine kinase signaling in development and cancer. Cytokine Growth Factor Rev 2005; 16: 441-67.

[11] Murakumo Y, Jijiwa M, Asai N, Ichihara M, Takahashi M. RET and neuroendocrine tumors. Pituitary 2006; 9: 179-92.

[12] Airaksinen MS, Saarma M. The GDNF family: signalling, biological functions and therapeutic value. Nat Rev Neurosci 2002; 3: 383-94.

[13] Asai N, Jijiwa M, Enomoto A, et al. RET receptor signaling: dysfunction in thyroid cancer and Hirschsprung's disease. Pathol Int 2006; 56: 164-72.

[14] Gujral TS, van Veelen W, Richardson DS, et al. A novel RET kinase-beta-catenin signaling pathway contributes to tumorigenesis in thyroid carcinoma. Cancer Res 2008; 68: 1338-46.

[15] Cassinelli G, Favini E, Degl'Innocenti D, et al. RET/PTC1-driven neoplastic transformation and proinvasive phenotype of human thyrocytes involve Met induction and beta-catenin nuclear translocation. Neoplasia 2009; 11: 10-21.

[16] Kouvaraki MA, Shapiro SE, Perrier ND, et al. RET proto-oncogene: a review and update of genotype-phenotype correlations in hereditary medullary thyroid cancer and associated endocrine tumors. Thyroid 2005; 15: 531-44.

[17] Raue F, Frank-Raue K. Genotype-phenotype relationship in multiple endocrine neoplasia type 2. Implications for clinical management. Hormones 2009; 8: 23-8.

[18] Corsello SM, Lovicu RM, Migneco MG, Rufini V, Summaria V. Diagnostic approach, genetic screening and prognostic factors of medullary thyroid carcinoma. Rays 2000; 25: 257-66.

[19] Machens A, Ukkat J, Brauckhoff M, Gimm O, Dralle, H. Advances in the management of hereditary medullary thyroid cancer. J Intern Med 2005; 257: 50-9.

[20] Sakorafas GH, Friess H, Peros G. The genetic basis of hereditary medullary thyroid cancer: clinical implications for the surgeon, with a particular emphasis on the role of prophylactic thyroidectomy. Endocr Relat Cancer 2008; 15: 871-84.

[21] Asai N, Iwashita T, Matsuyama M, Takahashi M. Mechanism of activation of the ret proto-oncogene by multiple endocrine neoplasia 2A mutations. Mol Cell Biol 1995; 15: 1613-9

[22] Santoro M, Carlomagno F, Romano A, et al. Activation of RET as a dominant transforming gene by germline mutations of MEN2A and MEN2B. Science 1995; 267: 381-3.

[23] Bocciardi R, Mograbi B, Pasini B, et al. The multiple endocrine neoplasia type 2B point mutation switches the specificity of the Ret tyrosine kinase towards cellular substrates that are susceptible to interact with Crk and Nck. Oncogene 1997; 15: 2257-65.

[24] Salvatore D, Melillo RM, Monaco C, et al. Increased in vivo phosphorylation of ret tyrosine 1062 is a potential pathogenetic mechanism of multiple endocrine neoplasia type 2B. Cancer Res 2001; 61: 1426-31.

[25] Manié S, Santoro M, Fusco A, Billaud M. The RET receptor: function in development and dysfunction in congenital malformation. Trends Genet 2001; 17: 580-9.

[26] Fattorusso O, Quadro L, Libroia A, et al. A GTG to ATG novel point mutation at codon 804 in exon 14 of the RET proto-oncogene in two families affected by familial medullary thyroid carcinoma. Hum Mut 1998; 1: S167-71

[27] Niccoli-Sire P, Murat A, Rohmer V, et al. Familial medullary thyroid carcinoma with noncysteine ret mutations: phenotype-genotype relationship in a large series of patients. J Clin Endocrinol Metab 2001; 86: 3746-53

[28] Eng C, Mulligan LM, Healey CS, et al. Heterogeneous mutation of the RET proto-oncogene in subpopulations of medullary thyroid carcinoma. Cancer Res 1996; 56: 2167-70.

[29] Elisei R, Cosci B, Romei C, et al. Prognostic significance of somatic RET oncogene mutations in sporadic medullary thyroid cancer: a 10-year follow-up study. J Clin Endocrinol Metab 2008; 93: 682-7.

[30] Moura MM, Cavaco BM, Pinto AE, et al. Correlation of RET somatic mutations with clinicopathological features in sporadic medullary thyroid carcinomas. Br J Cancer 2009; 100: 1777-83.

[31] Ameur N, Lacroix L, Roucan S, et al. Aggressive inherited and sporadic medullary thyroid carcinomas display similar oncogenic pathways. Endocr Relat Cancer 2009; 16: 1261-72.

[32] Moley JF, Brother MB, Wells SA, Spengler BA, Biedler JL, Brodeur GM. Low frequency of ras gene mutations in neuroblastomas, pheochromocytomas, and medullary thyroid cancers. Cancer Res 1991; 51: 1596-99.

[33] Horie H, Yokogoshi Y, Tsuyuguchi M, Saito S. Point mutations of ras and Gs alpha subunit genes in thyroid tumors. Jpn J Cancer Res 1995; 86: 737-42.

[34] Bockhorn M, Frilling A, Kalinin V, Schröder S, Broelsch CE. Absence of H- and K-ras oncogene mutations in sporadic medullary thyroid carcinoma. Exp Clin Endocrinol Diabetes 2000; 108: 49-53.

[35] Xing M. BRAF mutation in thyroid cancer. Endocr Relat Cancer 2005; 12: 245-62.

[36] Goutas N, Vlachodimitropoulos D, Bouka M, Lazaris AC, Nasioulas G, Gazouli M. BRAF mutation in BRAF and K-RAS mutation in a Greek papillary and medullary thyroid carcinoma cohort. Anticancer Res 2008; 28: 305-8.

[37] Cerrato A, De Falco V, Santoro M. Molecular genetics of medullary thyroid carcinoma: the quest for novel therapeutic targets. J Mol Endocrinol 2009; 43: 143-55.

[38] Koch CA, Brouwers FM, Vortmeyer AO, et al. Somatic VHL gene alterations in MEN2-associated medullary thyroid carcinoma. BMC Cancer 2006; 6: 131.

[39] Huang SC, Koch CA, Vortmeyer AO, et al. Duplication of the mutant RET allele in trisomy 10 or loss of the wild-type allele in multiple endocrine neoplasia type 2-associated pheochromocytomas. Cancer Res 2000; 60: 6223-6.

[40]   Nelkin BD, de Bustros AC, Mabry M, Baylin SB. The molecular biology of medullary thyroid carcinoma. A model for cancer development and progression. JAMA 1989; 261: 3130-5.

[41]   Pitt SC, Chen H. The phosphatidylinositol 3-kinase/akt signaling pathway in medullary thyroid cancer. Surgery 2008; 144: 721-4.

[42]   Segouffin-Cariou C, Billaud M. Transforming ability of MEN2A-RET requires activation of the phosphatidylinositol 3-kinase/AKT signaling pathway. J Biol Chem 2000; 275; 3568-76.

[43]   Kunnimalaiyaan M, Vaccaro AM, Ndiaye MA, Chen H. Overexpression of the NOTCH1 intracellular domain inhibits cell proliferation and alters the neuroendocrine phenotype of medullary thyroid cancer cells. J Biol Chem 2006; 281: 39819-30.

[44]   Kunnimalaiyaan M, Vaccaro AM, Ndiaye MA, Chen H. Inactivation of glycogen synthase kinase-3beta, a downstream target of the raf-1 pathway, is associated with growth suppression in medullary thyroid cancer cells. Mol Cancer Ther 2007; 6: 1151-8.

[45]   Stadler G, Wieser M, Streubel B, et al. Low telomerase activity: possible role in the progression of human medullary thyroid carcinoma. Eur J Cancer 2008; 44: 866-75.

[46]   van Veelen W, Klompmaker R, Gloerich M, et al. P18 is a tumor suppressor gene involved in human medullary thyroid carcinoma and pheochromocytoma development. Int J Cancer 2009; 124: 339-45.

[47]   Cavalheiro BG, Junqueira CR, Brandão LG. Expression of matrix metalloproteinase 2 (MMP-2) and tissue inhibitor of metalloproteinase 2 (TIMP-2) in medullary thyroid carcinoma: prognostic implications. Thyroid 2008; 18: 865-71.

[48]   Arnaldi LA, Borra RC, Maciel RM, Cerutti JM. Gene expression profiles reveal that DCN, DIO1, and DIO2 are underexpressed in benign and malignant thyroid tumors. Thyroid 2005; 15: 210-21.

[49]   Souza Meyer EL, Dora JM, Wagner MS, Maia AL. Decreased type 1 iodothyronine deiodinase expression might be an early and discrete event in thyroid cell dedifferentation towards papillary carcinoma. Clin Endocrinol 2005; 62: 672-8.

[50]   Souza Meyer EL, Goemann IM, Dora JM, Wagner MS, Maia AL. Type 2 iodothyronine deiodinase is highly expressed in medullary thyroid carcinoma. Mol Cell Endocrinol 2008; 16: 16-22.

[51]   Petrangolini G, Cuccuru G, Lanzi C, et al. Apoptotic cell death induction and angiogenesis inhibition in large established medullary thyroid carcinoma xenografts by Ret inhibitor RPI-1. Biochem Pharmacol 2006; 72: 405–14.

[52]   Bunone G, Vigneri P, Mariani L, et al. Expression of angiogenesis stimulators and inhibitors in human thyroid tumors and correlation with clinical pathological features. Am J Pathol 1999; 155: 1967-76.

[53]   Lee S, Nakamura E, Yang H, et al. Neuronal apoptosis linked to EglN3 prolyl hydroxylase and familial pheochromocytoma genes: developmental culling and cancer. Cancer Cell 2005; 8: 155-67.

[54]   Jebreel A, England J, Bedford K, Murphy J, Karsai L, Atkin S. Vascular endothelial growth factor (VEGF), VEGF receptors expression and microvascular density in benign and malignant thyroid diseases. Int J Exp Pathol 2007; 88, 271-7.

[55]   Sharma SV, Settleman, J. Oncogene addiction: setting the stage for molecularly targeted cancer therapy. Gene Dev 2007; 21: 3214-31.

[56]   Kodama Y, Asai N, Kawai K, et al. The RET proto-oncogene: a molecular therapeutic target in thyroid cancer. Cancer Sci 2005; 96: 143-8.

[57]   Fagin JA. Genetics of papillary thyroid cancer initiation: implications for therapy. Trans Am Clin Climatol Assoc 2005; 116: 259-69.

[58]   Nikiforova MN, Nikiforov YE. Molecular genetics of thyroid cancer: implications for diagnosis, treatment and prognosis. Expert Rev Mol Diagn 2008; 8: 83-5.

[59]   Lanzi C, Cassinelli G, Nicolini V, Zunino F. Targeting RET for thyroid cancer therapy. Biochem Pharmacol 2009; 77: 297-309.

[60]   Taniguchi M, Uehara Y, Matsuyama M, Takahashi M. Inhibition of RET tyrosine kinase activity by herbimycin A. Biochem Biophys Res Commun 1993; 195: 208-14.

[61]   Cassinelli G, Lanzi C, Pensa T, et al. Clavilactones, a novel class of tyrosine kinase inhibitors of fungal origin. Biochem Pharmacol 2000; 59: 1539-47.

[62]   Panta GR, Nwariaku F, Kim LT. RET signals through focal adhesion kinase in medullary thyroid cancer cells. Surgery 2004; 136: 1212-7.

[63]   De Martimprey H, Bertrand JR, Fusco A, et al. siRNA nanoformulation against the ret/PTC1 junction oncogene is efficient in an in vivo model of papillary thyroid carcinoma. Nucleic Acids Res 2008; 36: e2.

[64]   You YN, Lakhani V, Wells SA Jr. New directions in the treatment of thyroid cancer. J Am Coll Surg 2007; 205: S45-8.

[65]   Carlomagno F, Vitagliano D, Guida T, et al. The kinase inhibitor PP1 blocks tumorigenesis induced by RET oncogenes. Cancer Res 2002; 62: 1077-82.

[66]   Carlomagno F, Vitagliano D, Guida T, et al. Efficient inhibition of RET/papillary thyroid carcinoma oncogenic kinases by 4-amino-5-(4-chloro-phenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2). J Clin Endocrinol Metab 2003; 88: 1897-902.

[67]   Carniti C, Perego C, Mondellini P, Pierotti MA, Bongarzone I. PP1 inhibitor induces degradation of RETMEN2A and RETMEN2B oncoproteins through proteosomal targeting. Cancer Res 2003; 63: 2234-43.

[68]   Carlomagno F, Guida T, Anaganti S, et al. Disease associated mutations at valine 804 in the RET receptor tyrosine kinase confer resistance to selective kinase inhibitors. Oncogene 2004; 23: 6056-63.

[69]   Melillo RM, Barone MV, Lupoli G, et al. Ret-mediated mitogenesis requires Src kinase activity. Cancer Res 1999; 59: 1120-6.

[70]   Strock CJ, Park JI, Rosen M, et al. CEP-701 and CEP-751 inhibit constitutively activated RET tyrosine kinase activity and block medullary thyroid carcinoma cell growth. Cancer Res 2003, 63: 5559-63.

[71]   Strock CJ, Park JI, Rosen DM, et al. Activity of irinotecan and the tyrosine kinase inhibitor CEP-751 in medullary thyroid cancer. J Clin Endocrinol Metab 2006; 91: 79-84.

[72]   Lanzi C, Cassinelli G, Pensa T, et al. Inhibition of transforming activity of the ret/ptc1 oncoprotein by a 2-indolinone derivative. Int J Cancer 2000; 85: 384-90

[73]   Cuccuru G, Lanzi C, Cassinelli G, et al. Cellular effects and antitumor activity of RET inhibitor RPI-1 on MEN2A-associated medullary thyroid carcinoma. J Natl Cancer Inst 2004; 96: 1006-14.

[74]   Herbst RS, Heymach JV, O'Reilly MS, Onn A, Ryan, AJ. Vandetanib (ZD6474): an orally available receptor tyrosine kinase inhibitor that selectively targets pathways critical for tumor growth and angiogenesis. Expert Opin Investig Drugs 2007; 16: 239-49.

[75]   Carlomagno F, Vitagliano D, Guida T, et al. ZD6474, an orally available inhibitor of KDR tyrosine kinase activity, efficiently blocks oncogenic RET kinases. Cancer Res 2002; 15: 7284-90.

[76]   Knowles PP, Murray-Rust J, Kjaer S, et al. Structure and chemical inhibition of the RET tyrosine kinase domain. J Biol Chem 2006; 281: 33577-87.

[77]   Holden SN, Eckhardt SG, Basser R, et al. Clinical evaluation of ZD6474, an orally active inhibitor of VEGF and EGF receptor signaling, in patients with solid, malignant tumors. Ann Oncol 2005; 16: 1391-7.

[78]   Dagher R, Johnson J, Williams G, Keegan P, Pazdur R. Accelerated approval of oncology products: a decade of experience. J Natl Cancer Inst 2004; 96: 1500-9.

[79]   de Groot JW, Plaza Menacho I, Schepers H, et al. Cellular effects of imatinib on medullary thyroid cancer cells harboring

multiple endocrine neoplasia Type 2A and 2B associated RET mutations. Surgery 2006; 139: 806-14.

[80] Frank-Raue K, Fabel M, Delorme S, Haberkorn U, Raue F. Efficacy of imatinib mesylate in advanced medullary thyroid carcinoma. Eur J Endocrinol 2007; 157: 215-20.

[81] de Groot JW, Zonnenberg BA, van Ufford-Mannesse PQ, *et al*. A phase II trial of imatinib therapy for metastatic medullary thyroid carcinoma. J Clin Endocrinol Metab 2007; 92: 3466-9.

[82] Carlomagno F, Anaganti S, Guida T, *et al*. BAY 43-9006 inhibition of oncogenic RET mutants. J Natl Cancer Inst 2006; 98: 326-34.

[83] Polverino A, Coxon A, Starnes C, *et al*. AMG 706, an oral, multikinase inhibitor that selectively targets vascular endothelial growth factor, platelet-derived growth factor, and kit receptors, potently inhibits angiogenesis and induces regression in tumor xenografts. Cancer Res 2006; 66: 8715-21.

[84] Schlumberger MJ, Elisei R, Bastholt L, *et al*. Phase II study of safety and efficacy of motesanib in patients with progressive or symptomatic, advanced or metastatic medullary thyroid cancer. J Clin Oncol 2009; 27: 3794-801.

[85] Sherman SI, Wirth LJ, Droz JP, *et al*. Motesanib diphosphate in progressive differentiated thyroid cancer. N Engl J Med 2008; 359, 31-42.

[86] Salgia R, Hong DS, Camacho LH, *et al*. A phase I dose – escalation study of the safety and pharmacokinetics (PK) of XL184, a VEGFR and MET kinase inhibitor, administered orally to patients (pts) with advanced malignancies. J Clin Oncol 2007; 25 (18 Suppl): 14031.

[87] Yano L, Shimura M, Taniguchi M, *et al*. Improved gene transfer to neuroblastoma cells by a monoclonal antibody targeting RET, a receptor tyrosine kinase. Hum Gene Ther 2000; 11: 995-1004.

[88] Bennasroune A, Fickova M, Gardin A, *et al*. Transmembrane peptides as inhibitors of ErbB receptor signaling. Mol Biol Cell 2004;15: 3464-74.

[89] Barbas AS, White RR. The development and testing of aptamers for cancer. Curr Opin Investig Drugs 2009; 10: 572-8.

[90] Cerchia L, Ducongé F, Pestourie C, *et al*. Neutralizing aptamers from whole-cell SELEX inhibit the RET receptor tyrosine kinase. PLoS Biol 2005; 3, e123.

[91] Drosten M, Frilling A, Stiewe T, Putzer BM. A new therapeutic approach in medullary thyroid cancer treatment: inhibition of oncogenic RET signaling by adenoviral vector-mediated expression of a dominant-negative RET mutant. Surgery 2002; 132: 991-7.

[92] Drosten M, Stiewe T, Putzer BM. Antitumor capacity of a dominant-negative RET proto-oncogene mutant in a medullary thyroid carcinoma model. Hum Gene Ther 2003; 14: 971-82.

[93] Parthasarathy R, Cote GJ, Gagel RF. Hammerhead ribozyme-mediated inactivation of mutant RET in medullary thyroid carcinoma. Cancer Res 1999; 59: 3911-4.

[94] Hennige AM, Lammers R, Höppner W, *et al*. Inhibition of Ret oncogene activity by the protein tyrosine phosphatase SHP1. Endocrinology 2001; 142: 4441-7.

[95] Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol 2005; 23: 1001-27.

[96] Cao Y. Tumor angiogenesis and molecular targets for therapy. Front Biosci 2009; 14: 3962-73.

[97] McDermott, D.F. Immunotherapy of metastatic renal cell carcinoma. Cancer 2009; 115 (10 Suppl): 2298-305.

[98] Soh EY, Eigelberger MS, Kim KJ, *et al*. Neutralizing vascular endothelial growth factor activity inhibits thyroid cancer growth *in vivo*. Surgery 2000; 128: 1059-65.

[99] Cohen EE, Rosen LS, Vokes EE, *et al*. Axitinib is an active treatment for all histologic subtypes of advanced thyroid cancer: results from a phase II study. J Clin Oncol 2008; 26: 4708-13.

[100] Younes MN, Yigitbasi OG, Park YW, *et al*. Antivascular therapy of human follicular thyroid cancer experimental bone metastasis by blockade of epidermal growth factor receptor

and vascular growth factor receptor phosphorylation. Cancer Res 2005; 65: 4716-27.

[101] Hoffmann S, Burchert A, Wunderlich A, *et al*. Differential effects of cetuximab and AEE 788 on epidermal growth factor receptor (EGF-R) and vascular endothelial growth factor receptor (VEGF-R) in thyroid cancer cell lines. Endocrine 2007; 31: 105-13.

[102] Schoenberger J, Grimm D, Kossmehl P, Infanger M, Kurth E, Eilles C. Effects of PTK787/ZK222584, a tyrosine kinase inhibitor, on the growth of a poorly differentiated thyroid carcinoma: an animal study. Endocrinology 2004; 145: 1031-8.

[103] Gomez-Rivera F, Santillan-Gomez AA, Younes MN, *et al*. The tyrosine kinase inhibitor, AZD2171, inhibits vascular endothelial growth factor receptor signaling and growth of anaplastic thyroid cancer in an orthotopic nude mouse model. Clin Cancer Res 2007; 13: 4519-27.

[104] Zatelli MC, Piccin D, Tagliati F, Bottoni A, Luchin A, Degli Uberti E. SRC homology-2-containing protein tyrosine phosphatase-1 restrains cell proliferation in human medullary thyroid carcinoma. Endocrinology 2005; 146: 2692-8.

[105] Kunnimalaiyaan M, Ndiaye M, Chen, H. Apoptosis-mediated medullary thyroid cancer growth suppression by the PI3K inhibitor LY294002. Surgery 2006; 140: 1009–15.

[106] Ludwig L, Kessler H, Wagner M, *et al*. Nuclear factor-kappaB is constitutively active in C-cell carcinoma and required for RET-induced transformation. Cancer Res 2001; 61: 4526–35.

[107] Sippel RS, Carpenter JE, Kunnimalaiyaan M, Chen H. The role of human achaete-scute homolog-1 in medullary thyroid cancer cells. Surgery 2003; 34: 866–71.

[108] Ezzat S, Huang P, Dackiw A, Asa SL. Dual inhibition of RET and FGFR4 restrains medullary thyroid cancer cell growth. Clin Cancer Res 2005; 11: 1336-41.

[109] Mitsiades CS, Mitsiades NS, McMullan CJ, *et al*. Inhibition of the insulin-like growth factor receptor-1 tyrosine kinase activity as a therapeutic strategy for multiple myeloma, other hematologic malignancies, and solid tumors. Cancer Cell 2004; 5: 221-30.

[110] Fenaux P, Raza A, Mufti GJ, *et al*. A multicenter phase 2 study of the farnesyltransferase inhibitor tipifarnib in intermediate- to high-risk myelodysplastic syndrome. Blood 2007; 109: 4158-63.

[111] Kurzrock R, Kantarjian HM, Cortes JE, *et al*. Farnesyltransferase inhibitor R115777 in myelodysplastic syndrome: clinical and biologic activities in the phase 1 setting. Blood 2003; 102: 4527-34.

[112] Hong D, Ye L, Gagel R, *et al*. Medullary thyroid cancer: targeting the RET kinase pathway with sorafenib/tipifarnib. Mol Cancer Ther 2008; 7: 1001-6.

[113] Park JI, Strock CJ, Ball DW, Nelkin BD. The Ras/Raf/MEK/extracellular signal-regulated kinase pathway induces autocrine-paracrine growth inhibition *via* the leukemia inhibitory factor/JAK/STAT pathway. Mol Cell Biol 2003; 23: 543-54.

[114] Harwood AJ, Plyte SE, Woodgett J, Strutt H, Kay RR. Glycogen synthase kinase 3 regulates cell fate in Dictyostelium. Cell 1995; 80: 139-48.

[115] Cohen P, Frame S. The renaissance of GSK3. Nat Rev Mol Cell Biol 2001; 2: 769-76.

[116] Liang MH, Chuang DM. Differential roles of glycogen synthase kinase-3 isoforms in the regulation of transcriptional activation. J Biol Chem 2006; 281: 30479-84.

[117] Liang MH, Chuang DM. Regulation and function of glycogen synthase kinase-3 isoforms in neuronal survival. J Biol Chem 2007; 282: 3904-17.

[118] Mandal M, Kim S, Younes MN, *et al*. The Akt inhibitor KP372-1 suppresses Akt activity and cell proliferation and induces apoptosis in thyroid cancer cells. Br J Cancer 2005; 92: 1899-905.

[119] Pierotti MA, Greco A. Oncogenic rearrangements of the NTRK1/NGF receptor. Cancer Lett 2006; 232: 90-8.

[120] Gimm O, Dziema H, Brown J, *et al*. Mutation analysis of NTRK2 and NTRK3, encoding 2 tyrosine kinase receptors, in

sporadic human medullary thyroid carcinoma reveals novel sequence variants. Int J Cancer 2001; 92: 70-4.

[121]   McGregor LM, McCune BK, Graff JR, *et al*. Roles of trk family neurotrophin receptors in medullary thyroid carcinoma development and progression. Proc Natl Acad Sci USA 1999; 96: 4540-5.

[122]   Surmacz E. Growth factor receptors as therapeutic targets: strategies to inhibit the insulin-like growth factor I receptor. Oncogene 2003; 22 : 6589-97.

[123]   Höppener JW, Steenbergh PH, Slebos RJ, *et al*. Expression of insulin-like growth factor-I and -II genes in rat medullary thyroid carcinoma. FEBS Lett 1987; 215: 122-6.

[124]   Wang Z, Chakravarty G, Kim S, *et al*. Growth-inhibitory effects of human anti-insulin-like growth factor-I receptor antibody (A12) in an orthotopic nude mouse model of anaplastic thyroid carcinoma. Clin Cancer Res 2006; 12: 4755-65.

[125]   Acevedo VD, Ittmann M, Spencer DM. Paths of FGFR-driven tumorigenesis. Cell Cycle 2009; 8: 580-8.

[126]   Kondo T, Ezzat S, Asa SL. Pathogenetic mechanisms in thyroid follicular-cell neoplasia. Nat Rev Cancer 2006; 6: 292-306.

[127]   Boelaert K, McCabe CJ, Tannahill LA, *et al*. Pituitary tumor transforming gene and fibroblast growth factor-2 expression: potential prognostic indicators in differentiated thyroid cancer. J Clin Endocrinol Metab 2003; 88: 2341-7.

[128]   Eggo MC, Hopkins JM, Franklyn JA, Johnson GD, Sanders DS, Sheppard MC. Expression of fibroblast growth factors in thyroid cancer. J Clin Endocrinol Metab 1995; 80: 1006-11.

[129]   de la Torre NJ, Buley I, Wass JA, Turner HE. Angiogenesis and lymphangiogenesis in thyroid proliferative lesions: relationship to type and tumour behaviour. Endocr Relat Cancer 2006; 13: 931-44

[130]   St Bernard R, Zheng L, Liu W, Winer D, Asa SL, Ezzat S. Fibroblast growth factor receptors as molecular targets in thyroid carcinoma. Endocrinology 2005; 146: 1145-53.

[131]   Kondo T, Zheng L, Liu W, Kurebayashi J, Asa SL, Ezzat S. Epigenetically controlled fibroblast growth factor receptor 2 signaling imposes on the RAS/BRAF/mitogen-activated protein kinase pathway to modulate thyroid cancer progression. Cancer Res 2007; 67: 5461-70.

[132]   Castellone MD, Carlomagno F, Salvatore G, Santoro M. Receptor tyrosine kinase inhibitors in thyroid cancer. Best Pract Res Clin Endocrinol Metab 2008; 22: 1023-38.

[133]   Adams J. The proteasome: a suitable antineoplastic target. Nat Rev Cancer 2004; 4: 349-60.

[134]   Gallel P, Pallares J, Dolcet X, *et al*. Nuclear factor-kappaB activation is associated with somatic and germ line RET mutations in medullary thyroid carcinoma. Hum Pathol 2008; 39: 994-1001.

[135]   Pacifico F, Mauro C, Barone C, *et al*. Oncogenic and anti-apoptotic activity of NF-kappa B in human thyroid carcinomas. J Biol Chem 2004; 279: 54610-9.

[136]   Richardson PG, Barlogie B, Berenson J, *et al*. A phase 2 study of bortezomib in relapsed, refractory myeloma. N Engl J Med 2003; 348: 2609-17.

[137]   Orlowski RZ, Kuhn DJ. Proteasome inhibitors in cancer therapy: lessons from the first decade. Clin Cancer Res 2008; 14: 1649-57.

[138]   Mitsiades CS, McMillin D, Kotoula V, *et al*. Antitumor effects of the proteasome inhibitor bortezomib in medullary and anaplastic thyroid carcinoma cells *in vitro*. J Clin Endocrinol Metab 2006; 91: 4013-21.

[139]   Greenblatt DY, Cayo MA, Adler JT, *et al*. Valproic acid activates Notch1 signaling and induces apoptosis in medullary thyroid cancer cells. (2008) Ann Surg., 247, 1036-1040

[140]   Ning L, Greenblatt DY, Kunnimalaiyaan M, Chen H. Suberoyl bis-hydroxamic acid activates Notch-1 signaling and induces apoptosis in medullary thyroid carcinoma cells. Oncologist 2008; 13: 98-104.

[141]   Ning L, Jaskula-Sztul R, Kunnimalaiyaan M, Chen H. Suberoyl bishydroxamic acid activates notch1 signaling and suppresses tumor progression in an animal model of medullary thyroid carcinoma. Am Surg Oncol 2008; 15: 2600-5.

[142]   Cohen MS, Hussain HB, Moley JF. Inhibition of medullary thyroid carcinoma cell proliferation and RET phosphorylation by tyrosine kinase inhibitors. Surgery 2002; 132: 960-7.

[143]   Castellone MD, Santoro M. Dysregulated RET signaling in thyroid cancer. Endocrinol Metab Clin North Am 2008; 37: 363-74.

[144]   Schlumberger M, Carlomagno F, Baudin E, Bidart JM, Santoro M. New therapeutic approaches to treat medullary thyroid carcinoma. Nat Clin Pract Endocrinol Metab 2008; 4: 22-32.

[145]   Cakir M, Grossman AB. Medullary thyroid cancer: molecular biology and novel molecular therapies. Neuroendocrinology 2009; 90: 323-48.