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RAS proto-oncogene in medullary thyroid carcinoma

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Abstract

Medullary thyroid carcinoma (MTC) is a rare malignancy originating from the calcitonin-secreting parafollicular thyroid C cells. Approximately 75% of cases are sporadic. Rearranged during transfection (*RET*) proto-oncogene plays a crucial role in MTC development. Besides *RET*, other oncogenes commonly involved in the pathogenesis of human cancers have also been investigated in MTC. The family of human *RAS* genes includes the highly homologous *HRAS*, *KRAS*, and *NRAS* genes that encode three distinct proteins. Activating mutations in specific hotspots of the *RAS* genes are found in about 30% of all human cancers. In thyroid neoplasias, *RAS* gene point mutations, mainly in *NRAS*, are detected in benign and malignant tumors arising from the follicular epithelium. However, recent reports have also described *RAS* mutations in MTC, namely in *HRAS* and *KRAS*. Overall, the prevalence of *RAS* mutations in sporadic MTC varies between 0–43.3%, occurring usually in tumors with WT *RET* and rarely in those harboring a *RET* mutation, suggesting that activation of these proto-oncogenes represents alternative genetic events in sporadic MTC tumorigenesis. Thus, the assessment of *RAS* mutation status can be useful to define therapeutic strategies in *RET* WT MTC. MTC patients with *RAS* mutations have an intermediate risk for aggressive cancer, between those with *RET* mutations in exons 15 and 16, which are associated with the worst prognosis, and cases with other *RET* mutations, which have the most indolent course of the disease. Recent results from exome sequencing indicate that, besides mutations in *RET*, *HRAS*, and *KRAS*, no other recurrent driver mutations are present in MTC.

RAS in human cancer

Introduction

RAS research begins in 1964 with an observation by Jennifer Harvey (1964) that a murine leukemia virus, obtained from a leukemic rat, induced sarcomas in newborn rodents. Three additional retroviruses were subsequently identified in 1967 (Kirsten–MSV) (Kirsten & Mayer 1967), 1974 (BALB–MSV) (Peters *et al.* 1974), and

1978 (Rasheed strain of rat sarcoma virus) (Rasheed *et al.* 1978), and later were found to carry *RAS* oncogenes.

For the Harvey and Kirsten strains, *ras* was the acronym chosen for rat sarcoma, because of their ability to cause these tumors. Their discoverers' names became the basis for distinguishing them: Harvey and Kirsten viral

ras genes, or H-ras and K-ras. The nucleotide sequences of the H-ras and K-ras oncogenes were not published until 1982 (Dhar et al. 1982, Tsuchida et al. 1982).

The molecular cloning of a human transforming gene from the EJ/T24 bladder carcinoma cell line (Goldfarb et al. 1982, Pulciani et al. 1982, Shih & Weinberg 1982) revealed that the molecular basis of HRAS gene activation was a single missense mutation in codon 12, which was also found in the viral H-ras and K-ras genes (Reddy et al. 1982, Tabin et al. 1982, Taparowsky et al. 1982). The mechanism of KRAS activation from lung and colon tumor cells proved also to be the mutation of codon 12 (Capon et al. 1983). In 1983, a new human transforming gene, not previously found in retroviruses, was identified (Hall et al. 1983, Shimizu et al. 1983) as a third member of the RAS gene family. This gene was discovered in neuroblastoma-derived DNA and was named NRAS.

In addition to mutations at codon 12, RAS mutations were later identified at codons 13 and 61. Mutations affecting other regions of the RAS genes have also been found, but at very low frequencies. Therefore, these three codons harbor about 92–98% of all RAS mutations in human cancer and thus represent the hotspots of RAS activation.

The recent identification of germline RAS mutations in a class of genetic syndromes affecting normal development (RASopathies) implicates the aberrant RAS signaling in other human disorders (Tidyman & Rauen 2009), and expands the use of anti-RAS drugs, originally designed as anti-cancer therapies, as obvious potential therapies for these distinct developmental disorders.

Mechanism of oncogenic activation

In humans, the three ubiquitously expressed RAS genes encode four distinct but highly homologous ∼21 kDa proteins (known as p21): HRAS, NRAS, KRAS4A, and KRAS4B (the last two are alternative splice variants of the KRAS gene). These proteins are GTPases that function as molecular switches in regulating pathways that are responsible for diverse cellular processes such as proliferation, differentiation, migration, and apoptosis (Boguski & McCormick 1993). RAS proteins serve as transducers of signals that connect cell surface receptors to intracellular effector pathways, by switching between 'on' and 'off' conformations, which are conferred by the binding of GTP and GDP respectively. The transition between these two states, under physiological conditions, is regulated both by guanine nucleotide exchange factors, which induce RAS protein activation by promoting GDP for GTP

exchange, and by GPTase-activating proteins, which enhances RAS-mediated GTP hydrolysis. The major effect of the most common somatic mutations present in the oncogenic variants of RAS alleles is the maintenance of RAS in the GTP-bound state, with the consequent constant activation of a variety of RAS-dependent downstream effector pathways (Pylayeva-Gupta et al. 2011).

Incidence of somatic RAS mutations in human cancer

Oncogenic activation of RAS proteins due to missense mutations is frequently detected in several types of cancer. Different databases have collected all the existing information about specific mutations of RAS genes in different forms of human tumors. For instance, the Wellcome Trust Sanger Institute has launched a catalogue of somatic mutations in cancer (COSMIC) at http://sanger.ac.uk/cosmic, the most comprehensive database on human tumor mutations currently available (Forbes et al. 2011).

Based on the data obtained from the COSMIC dataset, 30.1% of all human tumors screened carry some mutation in any of the common RAS genes. KRAS is by far the most frequently mutated isoform, with oncogenic KRAS mutations being detected in 21.2% of all tumors analyzed (29 557 mutated samples of 139 474 samples tested). The frequency of somatic mutations concerning the other family members is much lower, namely 5.7 and 3.2% of samples screened for NRAS (3587 mutated samples of 62 609 samples tested) and HRAS (1127 mutated samples of 35 366 samples tested) respectively. However, these values are biased, because colorectal cancer, where KRAS mutations predominate, represents most of the cases (Prior et al. 2012). So when all cancer types are given equal weightings, the average pan-RAS mutation incidence is only 14.4% (34 271 mutated samples of 237 449 samples tested).

KRAS mutations are predominant in pancreatic tumors (57.3%) and significantly high percentages are also reported in tumors of the large intestine (34.7%), peritoneum (28.3%), biliary tract (25.2%), small intestine (19.5%), lung (16.9%), endometrium (15.2%), ovary (12.4%), and cervix (7.3%). HRAS mutations are most frequently detected in tumors of the skin (10.0%), salivary gland (9.4%), urinary tract (9.3%), cervix (8.4%), upper aerodigestive tract (7.9%), and penis (7.1%). By contrast, NRAS mutations have higher incidence in tumors of the skin (15.4%), hematopoietic malignancies (9.6%), tumors of the meninges (7.2%), and in thyroid carcinomas (6.2%).

Oncogenic mutations are concentrated within two hotspots (codons 12/13 and 61, which participate in the

GTP binding domain of the protein) for all *RAS* family members. However, the incidence of mutation at both sites varies among the three different main RAS isoforms. For *KRAS*, the majority of activating mutations are located at codons 12 and 13, which account for 96.3% of the mutations detected (81.9 and 14.4% respectively), whereas mutations affecting codon 61 account for only 1.6%. Although the biological and prognostic significance of other mutations described along this oncogene locus are largely unknown, mutations in exon 4 of *KRAS* were found to occur commonly and to predict a more favorable clinical outcome in patients with colorectal cancer (Janakiraman *et al.* 2010). Another work identified a novel *KRAS* co-mutation that altered codons 19 and 20, resulting in transitions at both codons (p.Leu19Phe/p.Thr20Ala) in the same allele, in a human colorectal cancer, and demonstrated that co-mutation of these codons is functionally significant (Naguib *et al.* 2011). Activating mutations of *NRAS* show a different distribution pattern, with 60.9% of the mutations occurring at codon 61 and a lower percentage of mutations found at codons 12 (23.4%) and 13 (11.4%). Finally, *HRAS* mutations follow another specific pattern, with the highest rates of mutation observed in codon 12 (36.7%), followed by codon 61 (34.9%) and codon 13 (20.6%).

Other alternative mechanisms, such as gene amplification, may also lead to RAS activation and contribute to the development of human neoplasia (Pulciani *et al.* 1985).

Specific patterns of *RAS* mutations in thyroid tumors derived from the follicular epithelium

In 1988, activating mutations of all three *RAS* oncogenes were first described in thyroid tumors (Lemoine *et al.* 1988, Suárez *et al.* 1988). Early reports demonstrated that the overall rate of *RAS* mutation in papillary carcinomas was significantly lower than in follicular carcinomas (Wright *et al.* 1989). Moreover, the frequency of *RAS* oncogene activation was similar in benign and malignant thyroid neoplasms, supporting the argument that mutation of *RAS* oncogenes is an early event in thyroid tumorigenesis (Namba *et al.* 1990). Subsequent studies on *RAS* oncogenes in thyroid tumors showed that most of the published series reported distinct results concerning the incidence of mutations, isoform pattern and correlation of mutations with histology (Vasko *et al.* 2003).

RAS mutations are found in a distinct percentage of thyroid cancers (Table 1). As reported in the current COSMIC somatic mutation database, *NRAS* mutations are

Table 1 Distribution and frequency of somatic *RAS* mutations in human malignant thyroid tumors

Thyroid tumor type	HRAS	KRAS	NRAS
Anaplastic carcinoma	4.6% (22/476)	8.1% (38/471)	15.4% (72/467)
Follicular carcinoma	6.3% (31/495)	3.9% (19/490)	15.7% (79/502)
Medullary carcinoma	9.3% (52/560)	3.0% (17/561)	0.6% (3/537)
Papillary carcinoma	1.8% (44/2416)	1.2% (36/2948)	4.2% (136/3210)

Data obtained from the catalogue of somatic mutations in cancer (COSMIC) at http://sanger.ac.uk/cosmic (accessed May 2014). Values are presented as the total percentage of samples mutated (total mutated samples/total samples tested) for that particular tumor type.

the most frequent *RAS* mutations in thyroid tumors (6.2%), followed by mutations in *HRAS* (3.9%) and *KRAS* (2.0%). *RAS* mutations have been detected in 40–50% of follicular carcinomas and 20–40% of follicular adenomas, and in oncocytic tumors but at lower frequency (Albarel *et al.* 2012). Several studies report that *RAS* mutations are a marker for aggressive thyroid cancer behavior and poor prognosis (Garcia-Rostan *et al.* 2003). In a recent report, *RAS* mutational analysis was performed in a series of 26 anaplastic thyroid carcinomas (ATCs) and 22 poorly differentiated thyroid carcinomas (PDTCs). In this study, *NRAS* was the most frequently mutated *RAS* oncogene isoform (26.9% in ATC and 18.2% in PDTC) and *HRAS* mutations were only present in ATC (3.8%), and no mutations were observed in *KRAS* either in ATC or PDTC (Pita *et al.* 2014).

RAS mutations have also been detected in medullary thyroid carcinomas (MTCs), and this subject will be further discussed in section 'RAS mutations in MTC'.

Medullary thyroid carcinoma

MTC is a neuroendocrine tumor originating from the calcitonin-producing neural crest-derived parafollicular C cells of the thyroid. They are named C cells due to their calcitonin hormone secretion and account for up to 1% of thyroid cells. These cells are mostly located in the posterior upper third of the lateral lobes of the thyroid gland, where the majority of MTC are found, and they also produce carcinoembryonic antigen (Stamatakos *et al.* 2011).

MTC was first described by Jaquet (1906) as 'malignant goiter with amyloid'. Hazard *et al.* (1959) characterized the medullary carcinoma and recommended its recognition as a distinct clinicopathological entity. Williams (1966)

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suggested that this tumor is derived from the parafollicular cells. MTC is a relatively rare carcinoma, representing 5–10% of all thyroid cancers, and accounts for up to 14% of all thyroid cancer-related deaths (Roman et al. 2006), with ∼1000 new diagnoses in the USA each year (Pacini et al. 2010).

MTC typically grows slowly and metastasizes to cervical and mediastinal nodal chains in up to 50% of cases. It also metastasizes to distant organs such as the lungs, liver, and bones in 20% of cases (Maliszewska et al. 2013). MTC is classically managed with surgery and, unlike DTC, MTC is not iodine avid and treatment with radioactive iodine is not indicated. Moreover, the prognosis of MTC is intermediate between well-DTC and ATC, being less favorable than that of papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma.

The 10-year overall survival rate of MTC is about 75% (Hundahl et al. 1998), but it decreases to 40% or less in patients with locally advanced or metastatic disease (Wells et al. 2012). Important prognostic factors that predict adverse outcome include advanced age at diagnosis, extent of primary tumor, nodal disease, and distant metastases (American Thyroid Association Guidelines Task Force et al. 2009). The occurrence of locoregional and distant metastases occurs preferentially within the first 5 years (Peixoto Callejo et al. 2006). There has been limited success in the use of systemic chemotherapy or radiotherapy to treat patients with metastatic MTC (Martins et al. 2006). Surgical removal of all malignant tissue performed at an early stage, when the disease is confined to the thyroid gland, is the only potentially curative treatment, and management guidelines for treatment of MTC were published by the American Thyroid Association (American Thyroid Association Guidelines Task Force et al. 2009).

MTC occurs in either sporadic (75%) or hereditary (25%) form, and is transmitted in an autosomal dominant pattern with a nearly 100% level of penetrance (Ponder et al. 1988), either as familial MTC without other endocrinopathies or as part of the multiple endocrine neoplasia (MEN) syndromes types 2A and 2B. The most aggressive variant of MTC is MEN2B. Since the seminal description by Wolfe et al. (1973), C-cell hyperplasia (CCH) has become recognized as a precursor to malignant transformation. Individuals affected with inherited MTC develop initially primary CCH that progresses to early invasive medullary microcarcinoma, and eventually evolves into grossly invasive macroscopic MTC. Familial forms often appear as multifocal and bilateral tumors developing at a young age (Schulten et al. 2011), whereas

in sporadic cases, the tumor is usually unifocal and discovered in the fifth or sixth decade of life (Heshmati et al. 1997). At diagnosis, patients with sporadic MTC most commonly present with an isolated thyroid nodule or a palpable cervical lymph node.

The rearranged during transfection (RET) proto-oncogene is the susceptibility gene for hereditary MTC. The RET gene was first identified in 1985 (Takahashi et al. 1985), and in 1993 and 1994 it was demonstrated that MEN2 syndromes were caused by germline RET mutations (Donis-Keller et al. 1993, Eng et al. 1994). Thus, constitutively activating RET point mutations occurring germline are present in virtually all patients with inherited MTC (Kouvaraki et al. 2005). The RET gene, composed of 21 exons, is mapped to chromosome 10q11.2 and encodes a tyrosine kinase receptor with a cysteine-rich extracellular domain, a transmembrane domain and an intracellular portion containing two tyrosine kinase domains. The RET gene plays a crucial role in regulating cell proliferation, migration, differentiation, and survival (Eng 1999).

RET mutations involved in MTC are gain-of-function alterations that increase RET kinase activity, resulting in a constant activation of several intracellular signaling cascades that lead ultimately to tumor growth. Several germline mutations of this gene have been associated with hereditary MTC and a strong genotype–phenotype correlation has been reported, with mutations in exons 10 or 11 occurring mainly in individuals with MEN2A and the p.Met918Thr mutation in exon 16 being found in the majority of MEN2B cases (Maliszewska et al. 2013). The presence of specific RET mutations determines the age of presentation and aggressiveness of the tumor (Cote & Gagel 2003). Point mutations in the RET gene cluster in exons 5, 8, 10, 11, 13–16, and exons 5 and 8 have been added recently to the routine panel of studied exons in MTC (Romei et al. 2011). Identification of a RET germline mutation is the definitive method to distinguish sporadic from inherited forms. RET molecular analysis is essential in MTC management, since early diagnosis improves prognosis and allows genetic screening and recommendations for prophylactic thyroidectomy in familial cases (American Thyroid Association Guidelines Task Force et al. 2009).

Although much is known about hereditary MTC, the oncogenic mechanisms underlying sporadic MTC are not so well characterized. Molecular studies have demonstrated the involvement of RET proto-oncogene in 12–100% of the sporadic forms of MTC, depending on the reported series (Moura et al. 2009). p.Met918Thr RET mutation is the most common mutation in sporadic MTC

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but its detection rate varies greatly (5–66%) in the published literature (Moura *et al.* 2009).

Somatic *RET* mutations have been correlated with a worse outcome (Ciampi *et al.* 2013) and associated with larger tumors, lymph node and distant metastases, more advanced stage at diagnosis and low overall survival, as well as male gender and young age at diagnosis (Dvorakova *et al.* 2008, Elisei *et al.* 2008, Mian *et al.* 2011). However, there are studies reporting no statistically significant association between clinical and pathological characteristics, and the presence or absence of somatic *RET* mutations (Tamburrino *et al.* 2012, Lyra *et al.* 2014).

In a recent study (Moura *et al.* 2009), somatic *RET* mutations were correlated with clinicopathological features of sporadic MTC and the results suggested a stratification of sporadic MTC patients into risk levels: patients with mutations in *RET* exons 15 and 16 were at the highest risk for aggressive MTC, followed by those having no detectable *RET* mutation, at intermediate risk, and patients bearing other *RET* mutations, who presented the lowest risk for a worse clinical outcome. Therefore, this study showed that *RET* mutations in exons 15 and 16 are associated with a more aggressive behavior than other *RET* mutations, as it has been shown *in vitro*, as well as in the hereditary variants of MTC.

Molecular alterations beyond *RET* mutations in MTC

Copy number alterations

Fluorescence *in situ* hybridization and real-time quantitative PCR revealed *RET* copy number alterations (CNA) in 27.7% MTC, represented either by *RET* gene amplification (exclusively in hereditary MTC) or chromosome 10 aneuploidy (more frequently observed in sporadic cases). These alterations were present in a variable percentage of cancer cells, suggesting a certain degree of tumoral heterogeneity in MTC. Moreover, a significant higher prevalence of *RET* CNA was observed in *RET* mutated MTC and correlated with a poor prognosis (Ciampi *et al.* 2012). Using array comparative genomic hybridization in primary tumors and metastases, it was shown that most MTC have only a few copy number changes (most commonly losses of chromosomes 1p, 4q, 19p, and 22q) and some (∼23%) do not even present any chromosomal gains and losses, indicating that, unlike most other tumor types (Pinkel & Albertson 2005), copy number changes in MTC are relatively rare (Flicker *et al.* 2012).

Gene and protein expression profile

Concerning protein expression profiling, one study assessed nuclear factor-kB (NF-kB) in a series of MTC in correlation with *RET* mutation status. NF-kB is a transcription factor implicated in a wide variety of cellular processes including cell growth, differentiation, and apoptosis and is known to be activated through several signaling pathways that involve growth factor receptors. NF-kB expression was more frequently altered in MTC with germline or somatic *RET* mutations than in cases without *RET* mutations, supporting the hypothesis that RET activation may be responsible for NF-kB overexpression in MTC (Gallel *et al.* 2008).

Sponziello *et al.* (2014) analyzed the expression levels of several genes involved in the epigenetic control of transcription by TaqMan low density arrays in a series of 54 MTC (13 familial and 41 sporadic, 33 carrying a *RET* mutation, and 13 a *RAS* somatic mutation). In this study, only overexpression of the histone acetyltransferases *KAT2A* and *KAT2B* and of histone demethylase *KDM5B* was observed in *RAS*-mutated compared to WT tumors. By contrast, *RET*-mutated tumors (both germline and somatic) showed higher transcript levels of many epigenetic regulators than both WT and *RAS*-mutated MTC; significant differences were detected in the expression levels of *HDAC3*, *6*, *7*, *8* and *10*, *EP300*, *KAT2A* and *B*, *SMYD4*, *CARM1*, *EHMT1*, *KDM1A*, *2A*, *4A*, *4C* and *5B*, *DNMT1* and *3A*. In the more aggressive MTC cases (i.e., occurrence of lymph node and distant metastases, persistent disease after primary treatment and disease-related death), the expression profiling revealed a significant increase of the histone methyltransferases *EZH2* and *SMYD3* expression, but no significant correlation was found with *RET* or *RAS* mutational status; however, a comparison with type or risk level of *RET* mutations was not performed.

MicroRNA

Dysregulation in microRNA (miRNA) expression has recently been implicated in the pathogenesis of many types of human cancers, including MTC (Nikiforova *et al.* 2008). In a series of 19 MTC, a signature of three miRNA (miR-183, miR-375, and miR-9*) enabled to distinguish familial from sporadic cases (Abraham *et al.* 2011). Mian *et al.* (2012) analyzed the expression of nine miRNA (miR-21, miR-127, miR-154, miR-224, miR-323, miR-370, miR-9*, miR-183, and miR-375) in 34 sporadic MTC and correlated this expression with *RET* status. From the whole

set of nine miRNA, only miR-127 was significantly associated with *RET* status, with sporadic MTC carrying somatic *RET* mutations showing a lower upregulation of this miRNA than those with a WT *RET*. No relationships with *RAS* somatic mutation were observed.

The oncomiR miR-21 specifically targets the tumor suppressor gene *PDCD4*, and recent studies suggest that *PDCD4* is also regulated by protein kinase B (AKT) (Fassan *et al.* 2012). In a large series of 64 MTC (56 sporadic and eight familial), Pennelli *et al.* (2015) demonstrated significant PDCD4 nuclear downregulation together with miR-21 upregulation, thus confirming their previous results obtained in a small series of MTC (Mian *et al.* 2012), and showed also that miR-21/PDCD4 expression correlates with clinicopathological findings and prognosis. Although these authors were unable to detect any significant correlation between the miR-21/PDCD4 pathway and somatic *RET* or *RAS* mutation status, they found that the six *RAS*-positive cases in their series of MTC sporadic tumors had higher nuclear PDCD4 expression levels than the *RET*-positive/*RAS*-negative or *RET*/*RAS* WT patients. *RAS*-positive cases also revealed an intense reactivity pattern for phospho-AKT on western blot analysis, indicating a preferential activation of the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway. It has been shown that phosphorylation of PDCD4 by AKT causes nuclear translocation and inhibits tumor suppressor function of PDCD4 (Palamarchuk *et al.* 2005), suggesting that in *RAS*-positive tumors PDCD4 is hypofunctional due to the interference of AKT.

In a recent study, the expression of four main genes involved in miRNA biogenesis (*DROSHA*, *DICER*, *DGCR8*, and *XPO5*) was investigated in a series of 54 MTC analyzed for *RET* and *RAS* mutations (Puppin *et al.* 2014). Overexpression of *DICER*, *DGCR8*, and *XPO5* was observed only in *RET*-mutated tumors (familial or sporadic) compared to *RET* WT, while *RAS*-mutated MTC did not show significant differences with respect to non-mutated tumors. When MTC with *RET* and *RAS* mutations were compared, only *DGCR8* was significantly overexpressed in *RET*-mutated tumors.

Taken together, miRNA may be useful as prognostic markers and represent new potential targets for a RNA-based therapy in the treatment of MTC.

Gene mutations

Few non-*RET* molecular alterations have been reported in MTC (Fig. 1). Of note, mutations in *TP53* and *RB1* are highly uncommon or absent in MTC (Yana *et al.* 1992, Yoshimoto *et al.* 1992, Herfarth *et al.* 1997, Agrawal *et al.* 2013). Some studies showed the presence of somatic loss of function mutations in the cell cycle inhibitor *CDKN2C* (p18^{INK4C}) in about 10% of human MTC, coexisting with activating *RET* mutations, and concluded that loss of function of this tumor suppressor gene contributes to *RET*-induced MTC development (van Veelen *et al.* 2009); however, other authors did not find *CDKN2C* mutations in MTC samples (Cerrato *et al.* 2009). Our group also looked for mutations in *CDKN1B* (p18^{INK4C}) and *CDKN2C* (p27^{KIP1}) genes in *RET* WT sporadic MTC patients and,
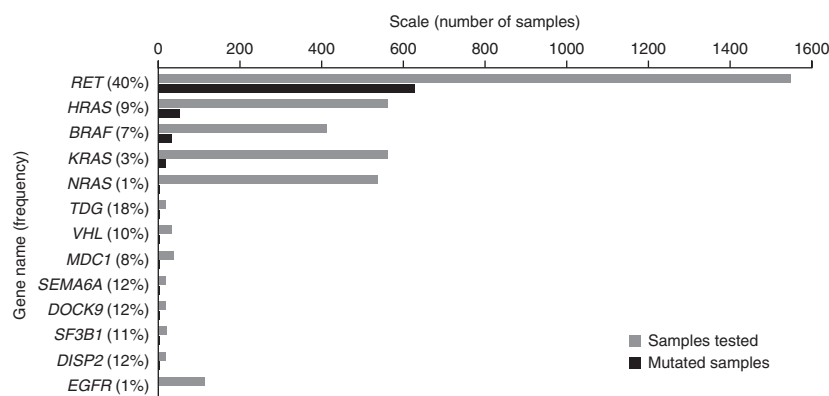

Figure 1

Summary of the most frequently mutated genes in MTC. The graph represents the genes that are mutated in at least two samples, or only in a single sample but after being tested in more than 100 MTC.

The data relate to the COSMIC v68 release, at http://sanger.ac.uk/cosmic (accessed May 2014).

except for an unreported germline *CDKN2C* variant (p.Pro117Ser) detected in one patient carrying a somatic *HRAS* mutation (p.Gly13Arg), no other mutations were found; no mutations were detected in *VHL* gene either in our series (M M Moura, B M Cavaco and V Leite, unpublished observations), although one (Koch *et al.* 2006) and two (Koperek *et al.* 2011) somatic *VHL* mutations have been previously described in hereditary and sporadic MTC respectively.

Many other oncogenes usually involved in the pathogenesis of various tumor types have been investigated in MTC with little success (Cerrato *et al.* 2009). MTC showed no genetic changes in c-*myc*, N-*myc*, and c-*erbB* proto-oncogenes (Yang *et al.* 1990), such as found commonly in human neoplasms, and absence of missense mutations of *Gsa* and *Gi2a* genes has also been observed in MTC (Horie *et al.* 1995). *EGFR* and *JAK2* mutations, genetic alterations that constitute potential therapeutic targets for drug-designed inhibitors in lung adenocarcinomas and myeloproliferative disorders, respectively, are infrequent or absent in MTC (Ameur *et al.* 2009). Similarly, mutations in *AKT1* and *CTNNB1* genes, which are key regulators of the PI3K/AKT/mTOR and canonical Wnt/β-catenin pathways, respectively, are virtually unknown in MTC (Schulten *et al.* 2011). Our group found no evidence of mutations in *AKT1*, *AKT2*, and *AKT3* within hotspots in sporadic MTC (M M Moura, B M Cavaco and V Leite, unpublished observations).

Point mutations in genes frequently implicated in thyroid tumorigenesis and linked to the RET pathway, such as *BRAF* and *PIK3CA*, have also been investigated in MTC in limited case series (Xing 2005, Ameur *et al.* 2009, Schlumberger *et al.* 2009). *PIK3CA* gene has been screened in 13 cases in a single study and neither mutations nor amplification were found (Wu *et al.* 2005). Our group confirmed that mutations in *PIK3CA*, within the classical hotspots, are absent in *RET* WT sporadic MTC patients (M M Moura, B M Cavaco and V Leite, unpublished observations). No *BRAF* gene mutations have been reported in three small series of 13 (Nikiforova *et al.* 2003), 14 (Xing *et al.* 2004), and 25 (Perren *et al.* 2004) MTC respectively. However, in a Greek cohort of 44 sporadic MTC, *BRAF* mutations were detected in 68.2% of the samples (Goutas *et al.* 2008), a finding that was not confirmed in the most recent studies (Schlumberger *et al.* 2009, Moura *et al.* 2011, Rapa *et al.* 2011, Schulten *et al.* 2011, Boichard *et al.* 2012, Agrawal *et al.* 2013, Nikiforova *et al.* 2013).

Recently, it was shown that mutations in the promoter region of *TERT* are relatively frequent in specific types of human cancers, including PTC and ATC (Vinagre *et al.* 2013); however, no mutations were detected in MTC (Killela *et al.* 2013, Vinagre *et al.* 2013), nor in normal thyroid and benign lesions (such as goiters, adenomas, or thyroiditis).

RAS mutations in MTC

The presence of *RAS* mutations in sporadic MTC is not entirely unexpected, since Johnston *et al.* (1998) demonstrated the development of MTC in *rascal* transgenic mice expressing v-Ha-*ras* under the control of the calcitonin/calcitonin gene-related peptide promoter. Also, the expression of transfected v-Ha-*ras* in MTC cell lines was previously shown to induce neuroendocrine differentiation *in vitro* (Nakagawa *et al.* 1987).

We performed sequencing analysis of the *RAS* genes in 66 sporadic MTC, 40 with *RET* mutation and 26 with *RET* WT, and somatic *HRAS* and *KRAS* mutations were detected in 15/26 (57.7%) and 3/26 (11.5%) of *RET* WT cases respectively (Moura *et al.* 2011; an additional sporadic MTC case was included in this series, M M Moura, B M Cavaco and V Leite, unpublished observations). Only 1/40 (2.5%) *RET*-positive cases had a somatic *RAS* mutation, namely in *HRAS* (p.Gly13Val). Overall, *RAS* mutations were present in 69.2% (18/26) of the *RET* WT cases and in only 2.5% of the *RET*-positive sporadic MTC ($P<0.0001$), suggesting that activation of the proto-oncogenes *RAS* and *RET* represents alternative genetic events in sporadic MTC tumorigenesis. These results were confirmed by other groups (Schlumberger *et al.* 2009, Schulten *et al.* 2011, Boichard *et al.* 2012, Mian *et al.* 2012, Tamburrino *et al.* 2012, Agrawal *et al.* 2013, Ciampi *et al.* 2013, Nikiforova *et al.* 2013, Lyra *et al.* 2014, Puppin *et al.* 2014, Simbolo *et al.* 2014, Sponziello *et al.* 2014, Pennelli *et al.* 2015). We found no other mutations in the entire coding region of the genes *HRAS*, *KRAS* and *RET* in the *RET* and *RAS* WT sporadic MTC (M M Moura, B M Cavaco and V Leite, unpublished observations).

A comprehensive review of the studies that were published in the literature concerning the prevalence of *RAS* point mutations in sporadic MTC is shown in Table 2. The prevalence of *RAS* mutations in such cases varies between 0–41.2 and 0–40.9% for *HRAS* and *KRAS*, respectively, and between 0–1.8% for *NRAS*, depending on the reported series.

The most frequent *HRAS* mutation is p.Gln61Arg ($n=35$), followed by p.Gln61Lys ($n=20$) and p.Gly13Arg ($n=12$), whereas the prevalent *KRAS* mutation is p.Gly12Arg ($n=13$). Since not all series analyzed all *RAS* isoforms, we present in Table 3 the overall prevalence of

Table 2 Prevalence of *RAS* point mutations in sporadic MTC

References	No. of cases	Method used		HRAS	KRAS	NRAS
Okazaki *et al.* (1989)	10	Slot blot analysis	Loci studied	Codons 12 and 61	Codons 12, 13, and 61	Codons 12 and 61
			Mutated samples	1/10 (10.0%)	0/10 (0.0%)	0/10 (0.0%)
			Mutation (no. of patients)	p.Gln61Arg (1)	–	–
Bockhorn *et al.* (2000)	15	Direct sequencing	Loci studied	Codons 12, 13, and 61	Codons 12, 13, and 61	NS
			Mutated samples	0/15 (0.0%)	0/15 (0.0%)	NS
			Mutation (no. of patients)	–	–	NS
Goutas *et al.* (2008)	44	Restriction fragment length poly-morphism analysis	Loci studied	NS	Codon 12	NS
			Mutated samples	NS	18/44 (40.9%)	NS
			Mutation (no. of patients)	NS	NA	NS
Schlumberger *et al.* (2009)	38	Direct sequencing	Loci studied	Exons 2 and 3	Exons 2 and 3	Exons 2 and 3
			Mutated samples	2/37[a] (5.4%)	1/38 (2.6%)	0/38 (0.0%)
			Mutation (no. of patients)	p.Gln61Arg (1) p.Gln61Lys (1)	p.Gly12Arg (1)	–
Moura *et al.* (2011)	65	Direct sequencing	Loci studied	Exons 2 and 3	Exons 2 and 3	Exons 2 and 3
			Mutated samples	15/65 (23.1%)	3/65 (4.6%)	0/65 (0.0%)
			Mutation (no. of patients)	p.Gly13Arg (5) p.Gln61Arg (5) p.Gln61Lys (3) p.Ala11_Gly12dup (1) p.Gly13Val (1)	p.Gln61Arg (1) p.Gln61Leu (1) p.Gln61Lys (1)	–
Rapa *et al.* (2011)	38	Pyrosequencing	Loci studied	Codon 61	Codons 12 and 13	Codon 61
			Mutated samples	0/38 (0.0%)	0/38 (0.0%)	0/38 (0.0%)
			Mutation (no. of patients)	–	–	–
Schulten *et al.* (2011)	9	Direct sequencing	Loci studied	Exons 2 and 3	Exons 2 and 3	Exons 2 and 3
			Mutated samples	1/9 (11.1%)	0/9 (0.0%)	0/9 (0.0%)
			Mutation (no. of patients)	p.Gly13Arg (1)	–	–
Tamburrino *et al.* (2012)	17	Exome sequencing	Loci studied	Coding region	Coding region	Coding region
			Mutated samples	4/17 (23.5%)	2/17 (11.8%)	0/17 (0.0%)
			Mutation (no. of patients)	p.Gln61Arg (2) p.Gly13Arg (1) p.Gln61Lys (1)	p.Gln61Leu (1) p.Gln61Lys (1)	–
Boichard *et al.* (2012)	30	Direct sequencing	Loci studied	Exons 2, 3, and 4	Exons 2, 3, and 4	Exons 2, 3, and 4
			Mutated samples	8/30 (26.7%)	5/30 (16.7%)	0/30 (0.0%)
			Mutation (no. of patients)	p.Gln61Arg (4) p.Gln61Lys (2) p.Gly13Arg (1) p.Lys117Asn (1)	p.Ala146Val (2) p.Gly12Arg (1) p.Gly12Val (1) p.Glu63Lys (1)	–
Ciampi *et al.* (2013)	175[b]	Direct sequencing	Loci studied	Exons 2 and 3	Exons 2 and 3	Exons 2 and 3
			Mutated samples	13/175 (7.4%)	4/175 (2.3%)	2[c]/175 (1.1%)
			Mutation (no. of patients)	p.Gln61Arg (5) p.Gln61Lys (3) p.Gln61Leu (2) p.Gly12Arg (1) p.Gly13Arg (1) p.Met72Ile (1)	p.Gly12Arg (3) p.Gln61His (1)	p.Gln61Arg (1) p.Gln61Leu (1)

Table 2 *Continued*

				Gene		
References	**No. of cases**	**Method used**		*HRAS*	*KRAS*	*NRAS*
Agrawal et al. (2013)	17[d]	Exome sequencing	Loci studied Mutated samples Mutation (no. of patients)	Coding region 0/17 (0.0%) –	Coding region 0/17 (0.0%) –	Coding region 0/17 (0.0%) –
	17[e]	Direct sequencing	Loci studied Mutated samples Mutation (no. of patients)	Exons 2, 3, 4, and 5 7/17 (41.2%) p.Gln61Arg (5) p.Gln61Lys (1) p.Lys117Asn (1)	Exons 2, 3, 4, and 5 2/17 (11.8%) p.Gly12Arg (1) p.Gly12Val (1)	NS NS NS
Nikiforova et al. (2013)	15	Next-generation sequencing	Loci studied Mutated samples Mutation (no. of patients)	Exons 2 and 3 3/15 (20.0%) p.Gln61Lys (2) p.Gly13Arg (1)	Exons 2 and 3 1/15 (6.7%) p.Gly12Arg (1)	Exons 2 and 3 0/15 (0.0%) –
Puppin et al. (2014)	41[f]	Direct sequencing	Loci studied Mutated samples Mutation (no. of patients)	Exons 2, 3, and 4 9/41 (22.0%) p.Gln61Lys (5) p.Gly13Arg (2) p.Gln61Arg (2)	Exons 2, 3, and 4 4/41 (9.8%) p.Gly12Arg (1) p.Gly12Val (1) p.Gln61Arg (1) p.Ala146Val (1)	Exons 2, 3, and 4 0/41 (0.0%) –
Simbolo et al. (2014)	20	Next-generation sequencing	Loci studied Mutated samples Mutation (no. of patients)	Exons 2 and 3[g] 3/20 (15.0%) p.Gln61Arg (3)	Exons 2, 3, and 4[g] 1/20 (5.0%) p.Gly12Arg (1)	Exons 2, 3, and 4[g] 0/20 (0.0%) –
Mancikova et al. (2014)	57	Direct sequencing	Loci studied Mutated samples Mutation (no. of patients)	Exons 2 and 3 10/57 (17.5%) NA	Exons 2 and 3 4/57 (7.0%) NA	Exons 2 and 3 0/57 (0.0%) –
Lyra et al. (2014)	77	Direct sequencing	Loci studied Mutated samples Mutation (no. of patients)	Exons 2 and 3 8/77 (10.4%) p.Gln61Arg (6) p.Gln61Lys (2)	Exons 2 and 3 3/77 (3.9%) p.Gly12Arg (2) p.Gly12Ser (1)	NS NS NS
Pennelli et al. (2015)	56	Direct sequencing	Loci studied Mutated samples Mutation (no. of patients)	Exons 2 and 3 3/56 (5.4%) p.Gly12Arg (1) p.Gln61Arg (1) p.Met72Ile (1)	Exons 2 and 3 2/56 (3.6%) p.Gly12Arg (2)	Exons 2 and 3 1/56 (1.8%) p.Gln61Leu (1)

NA, not available; NS, not studied.
[a]In one sample, *HRAS* mutation was not determined.
[b]Part of the series (*n*=34) was previously reported in another publication (Mian *et al*. 2012).
[c]Both patients with *NRAS* mutations had a PTC in association with the MTC.
[d]Discovery screen.
[e]Validation screen.
[f]This series of tumor samples was in part already analyzed in previous reports (Ameur *et al*. 2009, Boichard *et al*. 2012).
[g]Hotspot regions.

Table 3 Prevalence of *RAS* point mutations in studies where all three *RAS* isoforms were analyzed in sporadic MTC

References	No. of cases	Overall *RAS* mutations
Okazaki *et al*. (1989)	10	1/10 (10.0%)
Schlumberger *et al*. (2009)	38	3/38 (7.9%)
Moura *et al*. (2011)	65	18/65 (27.7%)
Rapa *et al*. (2011)	38	0/38 (0.0%)
Schulten *et al*. (2011)	9	1/9 (11.1%)
Tamburrino *et al*. (2012)	17	6/17 (35.3%)
Boichard *et al*. (2012)	30	13/30 (43.3%)
Ciampi *et al*. (2013)	175	19/175 (10.9%)
Agrawal *et al*. (2013)	17[a]	0/17 (0.0%)
Nikiforova *et al*. (2013)	15	4/15 (26.7%)
Puppin *et al*. (2014)	41	13/41 (31.7%)
Simbolo *et al*. (2014)	20	4/20 (20.0%)
Mancikova *et al*. (2014)	57	14/57 (24.6%)
Pennelli *et al*. (2015)	56	6/56 (10.7%)

[a]Discovery screen.

RAS mutations in the studies where all *RAS* isoforms were screened. As shown, the prevalence in these studies varies between 0–43.3%.

Boichard *et al*. (2012) screened the somatic mutational status of *RAS* genes in a series of 50 MTC, including 30 sporadic cases, and three mutations were detected in exon 4 both of *HRAS* (*n*=1, codon 117) and *KRAS* (*n*=2, codon 146). Mian *et al*. (2012) reported a p.Met72Ile mutation in exon 3 of the *HRAS* gene in a case with sporadic MTC.

There are several reasons that may explain the different prevalences of *RAS* point mutations that have been reported in MTC and that are summarized in Tables 2 and 3. First, in some of the studies, only the three mutational hotspots (codons 12, 13, and 61) were analyzed and/or not all *RAS* isoforms were screened. Secondly, the sizes of the published series differ substantially. Thirdly, different methodologies, with variable sensitivities for mutation detection, were used for the screening of *RAS* mutations. Fourthly, ethnic or environmental factors may also account for the reported differences in the prevalence of *RAS* mutations.

Agrawal *et al*. (2013) sequenced the exomes of 17 sporadic MTC and validated the frequency of all recurrently mutated genes and other genes of interest in an independent cohort of 40 MTC (21 hereditary and 19 sporadic). Whole-exome sequencing revealed that *RET* was somatically mutated in 12 sporadic MTC in the discovery screen, but no *HRAS* and *KRAS* mutations were detected. Other than *RET*, the genes that were mutated in at least two of the 17 MTC were *MDC1*, *SF3B1*, *MGAM*, *DOCK9*, *SEMA6A*, *TDG*, and *DISP2* (see Fig. 1). *RET*, *HRAS*, and *KRAS* genes were sequenced in additional 40 MTC (validation screen), and seven *HRAS* and two *KRAS* somatic mutations were detected. Thus, recent results from whole-exome sequencing indicate that MTC harbor relatively few mutations overall and suggest that there are no recurrent driver mutations other than *RET*, *HRAS*, and *KRAS* in these tumors.

Simbolo *et al*. (2014) examined the mutational status of 50 cancer-associated genes using a targeted next-generation sequencing (NGS) approach in a series of 20 sporadic MTC, previously analyzed for *RET* mutations by Sanger sequencing. Thirteen MTC harbored a somatic *RET* mutation; three of them, undetected by Sanger, were revealed by NGS, showing that targeted NGS has a higher sensitivity in the detection of mutations (these cases presented a proportion of *RET* mutated alleles below the 20% detection limit of Sanger analysis). One of the 13 *RET*-mutated cases also had a p.Phe354Leu germline mutation in *STK11*, which has been found in Peutz–Jeghers syndrome (Forcet *et al*. 2005). Four of the seven *RET* WT MTC carried a *RAS* mutation (three in *HRAS* and one in *KRAS*) and the three remaining cases were WT for all the 50 cancer-related genes. Thus, beside *RET*, *HRAS*, and *KRAS* mutations, no case exhibited somatic mutations in the other 47 genes studied.

As shown in Table 4, the prevalence of *RAS* mutations in different series of sporadic *RET* WT MTC varies between 0–81.3% (0–77.8, 0–31.3, and 0–2.8% for *HRAS*, *KRAS*, and *NRAS* respectively). *HRAS* is the gene most frequently affected, followed by *KRAS*, while mutations in *NRAS* remain a rare event. This contrasts with tumors arising from the thyroid follicular cells, where *NRAS* predominates (Vasko *et al*. 2003, Zhu *et al*. 2003). All the studies where *RET* and *RAS* were investigated in MTC showed mutual exclusivity between *RET* and *RAS* point mutations (Schlumberger *et al*. 2009, Moura *et al*. 2011, Schulten *et al*. 2011, Boichard *et al*. 2012, Mian *et al*. 2012, Tamburrino *et al*. 2012, Agrawal *et al*. 2013, Ciampi *et al*. 2013, Nikiforova *et al*. 2013, Lyra *et al*. 2014, Puppin *et al*. 2014, Simbolo *et al*. 2014, Sponziello *et al*. 2014, Pennelli *et al*. 2015). However, a somatic *HRAS* mutation was detected in one *RET*-positive case in the series of Moura *et al*. (2011), and to the best of our knowledge there are no other studies describing *RAS* mutations in *RET*-positive MTC. The two MTC patients who harbored *NRAS* mutations in the series of Ciampi *et al*. (2013) also presented a concomitant PTC.

RAS analyses performed in peripheral blood or normal thyroid tissue, from MTC cases harboring a *RAS* mutation in tumoral tissue, were negative indicating the somatic origin of the mutation. This result was expected, as germline *HRAS* mutations are associated with Costello's

Table 4 Prevalence of *RAS* point mutations in *RET*-negative sporadic MTC

References	No. of cases	Gene	Mutated samples (RAS+/RET−)	Overall *RAS* mutations
Schlumberger *et al.* (2009)	10	HRAS	2/10 (20.0%)	3/10 (30.0%)
		KRAS	1/10 (10.0%)	
		NRAS	0/10 (0.0%)	
Moura *et al.* (2011)	25	HRAS	14/25 (56.0%)	17/25 (68.0%)
		KRAS	3/25 (12.0%)	
		NRAS	0/25 (0.0%)	
Rapa *et al.* (2011)	18	HRAS	0/18 (0.0%)	0/18 (0.0%)
		KRAS	0/18 (0.0%)	
		NRAS	0/18 (0.0%)	
Schulten *et al.* (2011)	5	HRAS	1/5 (20.0%)	1/5 (20.0%)
		KRAS	0/5 (0.0%)	
		NRAS	0/5 (0.0%)	
Tamburrino *et al.* (2012)	10	HRAS	4/10 (40.0%)	6/10 (60.0%)
		KRAS	2/10 (20.0%)	
		NRAS	0/10 (0.0%)	
Boichard *et al.* (2012)	16	HRAS	8/16 (50.0%)	13/16 (81.3%)
		KRAS	5/16 (31.3%)	
		NRAS	0/16 (0.0%)	
Ciampi *et al.* (2013)	106	HRAS	13/106 (12.3%)	19/106 (17.9%)
		KRAS	4/106 (3.8%)	
		NRAS	2/106 (1.9%)	
Agrawal *et al.* (2013)	5[a]	HRAS	0/5 (0.0%)	9/14 (64.3%)
		KRAS	0/5 (0.0%)	
		NRAS	0/5 (0.0%)	
	9[b]	HRAS	7/9 (77.8%)	
		KRAS	2/9 (22.2%)	
		NRAS	NS	
Nikiforova *et al.* (2013)	8	HRAS	3/8 (37.5%)	4/8 (50.0%)
		KRAS	1/8 (12.5%)	
		NRAS	0/8 (0.0%)	
Puppin *et al.* (2014)	21	HRAS	9/21 (42.9%)	13/21 (61.9%)
		KRAS	4/21 (19.0%)	
		NRAS	0/21 (0.0%)	
Simbolo *et al.* (2014)	7	HRAS	3/7 (42.9%)	4/7 (57.1%)
		KRAS	1/7 (14.3%)	
		NRAS	0/7 (0.0%)	
Mancikova *et al.* (2014)	29	HRAS	10/29 (34.5%)	14/29 (48.3%)
		KRAS	4/29 (13.8%)	
		NRAS	0/29 (0.0%)	
Lyra *et al.* (2014)	41	HRAS	8/41 (19.5%)	11/41 (26.8%)
		KRAS	3/41 (7.3%)	
		NRAS	NS	
Pennelli *et al.* (2015)	36	HRAS	3/36 (8.3%)	6/36 (16.7%)
		KRAS	2/36 (5.6%)	
		NRAS	1/36 (2.8%)	

NS, not studied.
[a]Discovery screen.
[b]Validation screen.

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syndrome and germline *KRAS* mutations are responsible for Noonan's syndrome, which are developmental disorders caused by dysregulation of the RAS/RAF/MEK/ERK pathway (Tidyman & Rauen 2009).

It has already been demonstrated that the mTOR pathway is activated in sporadic and hereditary MTC (Rapa *et al.* 2011, Tamburrino *et al.* 2012) with a preferential expression in cases with germline *RET* mutations (Rapa *et al.* 2011). However, it remains unknown whether *RAS* mutations can also lead to the activation of this signaling pathway in MTC. Lyra *et al.* (2014) evaluated mTOR activation in a series of 87 MTC (ten familial and 77 sporadic), and *RAS* mutations were significantly associated with higher intensity of phospho-S6 ribosomal protein (p-S6) (a downstream effector of mTOR) expression; a significantly lower cytoplasmic expression of PTEN (a mTOR inhibitor) was observed in WT *RAS* cases in comparison with those with *RAS*

mutation. These results reveal an association between the activation of the mTOR pathway and the presence of *RAS* mutations in MTC, and this is in accordance with recent findings of Pennelli *et al.* (2015), as mentioned before (see section 'Molecular alterations beyond *RET* mutations in MTC').

Correlation of *RET* and *RAS* somatic mutations with clinicopathological features in sporadic MTC

It order to correlate *RET* and *RAS* mutations with clinical and pathological characteristics, our series of MTC patients (Moura *et al.* 2011) was divided into four groups (Table 5): group 1, with mutations in *RET* exons 15 and 16 ($n=24$, 36.4%) which includes somatic p.Met918Thr and p.Ala883Phe *RET* mutation cases, as patients with American Thyroid Association (ATA) level D mutations (codons 883 and 918) are at the highest risk for early development and growth of MTC (Frank-Raue *et al.* 2010); group 2, bearing other *RET* mutations ($n=15$, 22.7%); group 3, carrying a *RAS* mutation but no *RET* mutation ($n=18$, 27.3%); and group 4, having no detectable *RET* or *RAS* mutations ($n=8$, 12.1%). One *RAS*-positive/ *RET*-positive case ($n=1$, 1.5%) was excluded. Group 1 had higher prevalence of lymph node ($P=0.0014$) and distant ($P=0.017$) metastases, higher number of positive lymph nodes ($P=0.0011$), were more frequently associated with stage IV ($P=0.005$, vs stages I–III), and presented more often multifocal tumors ($P=0.008$), than cases with other *RET* mutations. Patients having no *RET* mutations (either *RAS*-positive or *RAS*-negative) were at intermediate risk and there was no statistically significant difference between *RAS*-positive and *RAS*-negative patients. In conclusion, these findings indicate that, among the sporadic MTC cases, patients with *RAS* mutations have an intermediate risk between those with ATA-D *RET* mutations, which are associated with the worst prognosis, and cases with other *RET* mutations, that have the most indolent course.

Besides our study, only a few others that performed mutational analysis (see Table 2) have correlated genotype with patient clinical findings.

Ciampi *et al.* (2013) correlated the *RAS* mutation status in *RET*-negative sporadic MTC with the clinical and pathological parameters (sex, age at diagnosis, T categories, size of tumor, lymph node metastases, distant metastases, stage, and status of the disease) of the patients and no statistically significant differences were observed. However, a higher but not statistically significant

prevalence of disease-free survival was found in the *RAS*-mutated group, suggesting that MTC harboring a *RAS* mutation represent a subgroup of tumors with a less aggressive behavior.

In the study of Simbolo *et al.* (2014), clinical follow-up and serum calcitonin levels indicated that at the end of follow-up seven of 12 *RET*-mutated MTC patients had relapsed (six of them harbored the p.Met918Thr mutation), while the four *RAS*-mutated cases were disease free; two of the three patients with MTC WT for all 50 genes also relapsed during the follow-up period. No significant association was observed between tumor recurrence and clinicopathological/molecular features. Thus, although not fully demonstrated, it seems that *RAS* is probably related to a less aggressive phenotype with a better outcome, and the detection of mutations by NGS may improve the diagnostic stratification of sporadic MTC.

Goutas *et al.* (2008) found no significant association between *KRAS* and *BRAF* mutations and clinicopathological parameters (age, gender, tumor size, stage, or nodal metastasis) in sporadic MTC. Similarly, in the study of Tamburrino *et al.* (2012) neither the presence nor the type of *RET* and *RAS* mutation was correlated with gender, age, histological variant (spindle cells, epithelioid), or other histological features (fibrosis, amyloid, or necrosis).

Lyra *et al.* (2014) attempted to correlate *RAS* and *RET* genotype results and clinicopathological data (gender, age, tumor size, nodal metastasis, invasion, or amyloid stroma) and did not disclose any associations with any clinicopathological feature for *RAS*-mutated cases; except for MTC patients displaying germline *RET* mutations that were younger than those with a somatic *RET* mutation or no *RET* mutation at all, no other significant association was observed between *RET* mutations and gender, tumor size, nodal metastasis, invasive features, or presence of amyloid stroma.

RAS mutations as a therapeutic target in MTC

Research over the last several years has enabled a good understanding of the genetic defects and altered molecular pathways that are involved in MTC development, and several promising therapeutic agents that target these specific alterations have been designed to treat advanced or metastatic MTC (Fig. 2) (Giunti *et al.* 2013, Haraldsdottir & Shah 2014).

Patients with progressive MTC have been treated in the last years with small molecule tyrosine kinase inhibitors (TKI), with remarkable results (Hu *et al.* 2014).

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Table 5 Correlation of *RET* and *RAS* mutations with the clinical and pathological characteristics of sporadic MTC

Characteristics	Group 1 p.Met918Thr and p.Ala883Phe *RET* mutation	Group 2 Other *RET* mutation	Group 3 No *RET* mutation *RAS* mutation	Group 4 No *RET* mutation No *RAS* mutation	P value
Sex					0.756[a]
Female	50.0% (12/24)	53.3% (8/15)	66.7% (12/18)	50.0% (4/8)	
Male	50.0% (12/24)	46.7% (7/15)	33.3% (6/18)	50.0% (4/8)	
Clinical presentation					0.050[a]
Thyroid nodule	37.5% (9/24)	100.0% (15/15)	64.7% (11/17)	50.0% (4/8)	
Lymph node	25.0% (6/24)	0.0% (0/15)	11.8% (2/17)	12.5% (1/8)	
Thyroid nodule and lymph node	37.5% (9/24)	0.0% (0/15)	23.5% (4/17)	37.5% (3/8)	
Age at surgery (years), mean ± s.e.m.	50.88 ± 2.82	59.40 ± 3.35	60.11 ± 3.77	51.75 ± 4.11	0.1113[b]
Tumor size (cm), mean ± s.e.m.	3.50 ± 0.41	2.81 ± 0.44	5.02 ± 0.81	3.00 ± 0.59	0.0542[b]
Postoperative serum calcitonin[c]					0.184[a]
Undetectable	9.1% (2/22)	33.3% (5/15)	25.0% (4/16)	37.5% (3/8)	
Detectable	90.9% (20/22)	66.7% (10/15)	75.0% (12/16)	62.5% (5/8)	
Serum calcitonin[c] at last control					0.076[a]
Undetectable	14.3% (3/21)	53.8% (7/13)	37.5% (6/16)	42.9% (3/7)	
Detectable	85.7% (18/21)	46.2% (6/13)	62.5% (10/16)	57.1% (4/7)	
T categories					0.378[a]
T1	12.5% (3/24)	50.0% (7/14)	17.6% (3/17)	14.3% (1/7)	
T2	37.5% (9/24)	21.4% (3/14)	29.4% (5/17)	42.9% (3/7)	
T3	16.7% (4/24)	21.4% (3/14)	17.6% (3/17)	14.3% (1/7)	
T4	33.3% (8/24)	7.1% (1/14)	35.3% (6/17)	28.6% (2/7)	
T categories grouping					0.265[a]
T1–T3	66.7% (16/24)	92.9% (13/14)	64.7% (11/17)	71.4% (5/7)	
T4	33.3% (8/24)	7.1% (1/14)	35.3% (6/17)	28.6% (2/7)	
Lymph node metastases					*0.0014*[d]
N1	87.5% (21/24)	26.7% (4/15)	66.7% (12/18)	75.0% (6/8)	
N0	12.5% (3/24)	73.3% (11/15)	33.3% (6/18)	25.0% (2/8)	
Distant metastases					*0.027*[a]
M1	43.5% (10/23)	0.0% (0/12)	25.0% (4/16)	14.3% (1/7)	
M0	56.5% (13/23)	100.0% (12/12)	75.0% (12/16)	85.7% (6/7)	
Stage					*0.029*[a]
I	4.2% (1/24)	38.5% (5/13)	11.1% (2/18)	12.5% (1/8)	
II	4.2% (1/24)	15.4% (2/13)	22.2% (4/18)	12.5% (1/8)	
III	4.2% (1/24)	15.4% (2/13)	5.6% (1/18)	0.0% (0/8)	
IV	87.5% (21/24)	30.8% (4/13)	61.1% (11/18)	75.0% (6/8)	
Stage grouping					*0.005*[a]
I–III	12.5% (3/24)	69.2% (9/13)	38.9% (7/18)	25.0% (2/8)	
IV	87.5% (21/24)	30.8% (4/13)	61.1% (11/18)	75.0% (6/8)	
Number of positive lymph nodes, mean ± s.e.m.	11.29 ± 2.01	1.20 ± 0.67	6.53 ± 1.68	6.00 ± 1.96	*0.0011*[e]
Follow-up (months), mean ± s.e.m.	87.68 ± 14.93	75.93 ± 14.81	74.36 ± 16.74	61.38 ± 25.85	0.8035[b]
Status at last control					0.087[a]
Disease free	14.3% (3/21)	53.3% (8/15)	35.3% (6/17)	28.6% (2/7)	
Non-disease free	85.7% (18/21)	46.7% (7/15)	64.7% (11/17)	71.4% (5/7)	
Presence of extraglandular extension	39.1% (9/23)	7.1% (1/14)	35.3% (6/17)	42.9% (3/7)	0.136[a]
Presence of vascular invasion	55.6% (10/18)	36.4% (4/11)	53.8% (7/13)	71.4% (5/7)	0.585[a]
Presence of multifocality	39.1% (9/23)	0.0% (0/13)	5.9% (1/17)	28.6% (2/7)	*0.008*[a]
Ploidy pattern					0.545[a]
Diploid	87.5% (21/24)	73.3% (11/15)	88.9% (16/18)	75.0% (6/8)	
Aneuploid	12.5% (3/24)	26.7% (4/15)	11.1% (2/18)	25.0% (2/8)	
S-phase fraction (%), mean ± s.e.m.	7.07 ± 0.88	6.08 ± 0.97	6.57 ± 0.89	6.94 ± 1.22	0.8901[b]

P values in italics and bold are statistically significant. One *RET*-positive sporadic MTC with a *RAS* mutation was excluded.
[a]Fisher's exact test.
[b]One-way ANOVA.
[c]Calcitonin values <2 ng/l were regarded as undetectable.
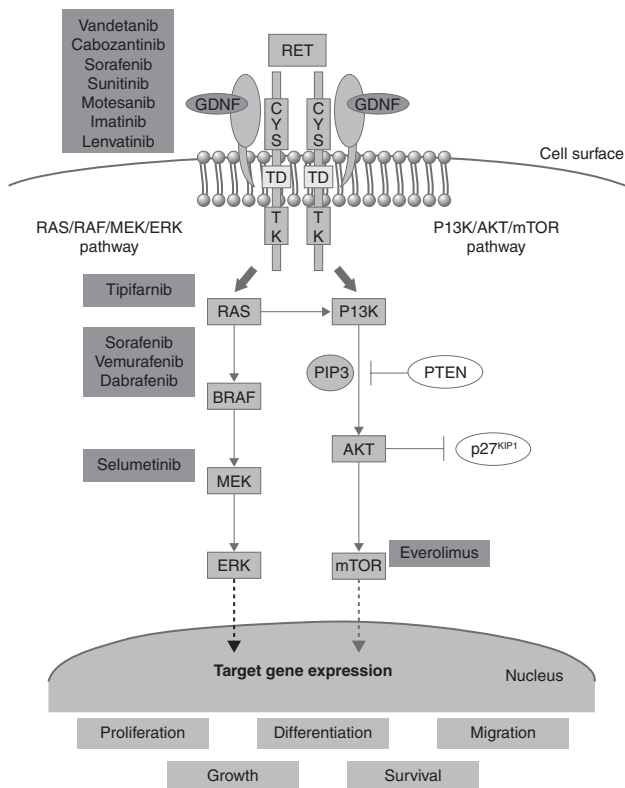[d]χ^2 test.
[e]Kruskal–Wallis test.

Figure 2

Signaling pathways implicated in thyroid carcinogenesis. RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways are involved in propagation of signals from cell membrane tyrosine kinase receptors into the nucleus and regulate multiple cellular processes, including proliferation, survival, growth, migration, and differentiation. The main therapeutic agents and their targets are shown. CYS, cysteine-rich domain; GDNF, glial cell-derived neurotrophic factor; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; TD, transmembrane domain; TK, tyrosine kinase domain.

Some studies are targeting the RAS pathway, e.g., through the use of farnesyltransferase inhibitors (FTI), which block the main post-translational modification of the RAS protein, thus interfering with its localization to the inner surface of the plasma membrane and subsequent activation of downstream effectors (Caponigro et al. 2003). Although initially developed as an approach to target RAS in cancer, FTI have later been recognized as acting by additional and more complex mechanisms, involving RhoB, centromere-binding proteins and probably other farnesylated proteins.

Hong et al. (2008) reported on a patient with sporadic MTC with metastatic disease, who was treated with a combination of sorafenib (RET and RAF kinase and vascular endothelial growth factor receptor inhibitor) and tipifarnib (inhibitor of RAS farnesylation), which resulted in a marked clinical response. In a phase I study,

it was shown that combining the multikinase inhibitor sorafenib with the FTI tipifarnib resulted in significant activity, particularly in patients with RET mutations (Hong et al. 2009). More recently, Hong et al. (2011) reported a phase I trial where 13 patients with MTC, eight of them (61.5%) carrying germline or somatic RET mutations, were treated with sorafenib combined with tipifarnib. MTC partial response rate was 38.5% (5/13) and stable disease of at least 6 months was 30.8% (4/13). Unfortunately, RAS mutation status was not analyzed in these studies.

Sherman et al. (2013a,b) investigated the association of RET and RAS mutations with efficacy outcomes in the phase III study of cabozantinib in MTC (Schöffski et al. 2012). Cabozantinib is a potent inhibitor of MET, VEGFR2, and RET (Hoy 2014). Sixteen of 85 tested patients (5% of total study patients) with WT or unknown RET status were found to harbor a RAS gene mutation and these patients presented a similar tumor response rate (31%) and progression-free survival (47 weeks) as RET-mutated patients (32% and 60 weeks).

Recently, it has been demonstrated that the expression of key TKI target proteins varies in MTC according to the specific RET mutation present (Rodríguez-Antona et al. 2014), a finding that could be used to improve the clinical response of MTC patients. However, the variable response to treatment with TKI remains largely unexplained and little is known about which patients would most benefit from a particular drug therapy. Mancikova et al. evaluated the influence of RAS mutations on the expression levels of eight key TKI targets (EGFR, KIT, MET, PDGFRB, VEGF, VEGFR1, VEGFR2, and VEGFR3) in a series of 84 molecularly characterized primary MTC tumors (27 familial and 57 sporadic). In contrast to RET-mutated tumors, RAS-positive MTC did not express MET and PDGFRB, and stained less frequently for VEGFR3; furthermore, WT tumors expressed VEGF more often than both RAS- and RET-mutated tumors (Mancikova et al. 2014). According to these authors, the differences in drug response observed in the study of Sherman et al. (2013a,b), namely longer progression-free survival for RET-mutated patients treated with cabozantinib when compared with RAS-mutated patients (60 weeks vs 47 weeks), could be explained, at least in part, by the differential expression of cabozantinib targets, as the RAS-mutated group expresses less often important targets of this drug.

These findings suggest that the assessment of RAS mutation status in sporadic RET-negative MTC can be useful to develop personalized targeted therapies.

In conclusion, analysis of *RET* and *RAS* mutations in sporadic MTC can be of value both for prognostic purposes and for therapeutic strategies.

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