# Exomic Sequencing of Medullary Thyroid Cancer Reveals Dominant and Mutually Exclusive Oncogenic Mutations in *RET* and *RAS*

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**Context:** Medullary thyroid cancer (MTC) is a rare thyroid cancer that can occur sporadically or as part of a hereditary syndrome.

**Objective:** To explore the genetic origin of MTC, we sequenced the protein coding exons of approximately 21,000 genes in 17 sporadic MTCs.

**Patients and Design:** We sequenced the exomes of 17 sporadic MTCs and validated the frequency of all recurrently mutated genes and other genes of interest in an independent cohort of 40 MTCs comprised of both sporadic and hereditary MTC.

**Results:** We discovered 305 high-confidence mutations in the 17 sporadic MTCs in the discovery phase, or approximately 17.9 somatic mutations per tumor. Mutations in *RET*, *HRAS*, and *KRAS* genes were identified as the principal driver mutations in MTC. All of the other additional somatic mutations, including mutations in spliceosome and DNA repair pathways, were not recurrent in additional tumors. Tumors without *RET*, *HRAS*, or *KRAS* mutations appeared to have significantly fewer mutations overall in protein coding exons.

**Conclusions:** Approximately 90% of MTCs had mutually exclusive mutations in *RET*, *HRAS*, and *KRAS*, suggesting that *RET* and *RAS* are the predominant driver pathways in MTC. Relatively few mutations overall and no commonly recurrent driver mutations other than *RET*, *HRAS*, and *KRAS* were seen in the MTC exome. **(*J Clin Endocrinol Metab* 98: E364–E369, 2013)**

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Abbreviations: dNTP, Deoxyribonucleotide triphosphate; EB, elution buffer; MEN 2, multiple endocrine neoplasia type 2; MTC, medullary thyroid cancer; PDAC, pancreatic ductal adenocarcinoma.

Medullary thyroid cancer (MTC) is a relatively uncommon type of thyroid cancer derived from the thyroid calcitonin-secreting parafollicular cell lineage. These tumors account for approximately 1200 new cancer diagnoses each year in the United States (~2% of the 56 500 new thyroid cancer cases in 2012) (1). Nevertheless, MTC accounts for a disproportionate fraction of thyroid cancer deaths. The overall 10-year survival for patients with MTC is approximately 60%, falling to approximately 40% if distant metastases are present at diagnosis (2). Patients typically die of widespread metastasis affecting liver, lung, and bone or locally aggressive disease with tracheal or esophageal invasion.

MTC can occur as part of the autosomal dominant hereditary syndrome multiple endocrine neoplasia type 2 (MEN 2). These hereditary cases account for approximately 25% of the total cases of MTC. In the germline DNA of patients with MEN 2, activating mutations in the *RET* gene are found in virtually all cases. *RET* encodes a receptor tyrosine kinase that normally binds a family of ligands including glial derived neurotrophic factor and is thought to provide growth and survival signaling via the RAF-MEK-ERK and PI3K-AKT-mTOR pathways (3). Activating mutations in *RET* can confer ligand-independent growth and resistance to apoptotic stimuli. Significantly, 30%–45% of sporadic MTC tumors also bear somatic *RET* mutations, the majority resembling those seen in the most aggressive hereditary form, MEN 2B (4). The somatic M918T *RET* mutation has proved to be a strong negative prognostic marker for overall and disease-free survival (4).

There is a profound unmet need for novel therapeutic approaches for MTC, both in the hereditary and sporadic settings. Chemotherapy and radiation therapy are relatively ineffective in MTC, and, until recently, there was no Food and Drug Administration-approved therapy for MTC. In 2011, the Food and Drug Administration approved the tyrosine kinase inhibitor vandetanib, which inhibits *RET*, as well as *VEGFR2* and *EGFR*, for treatment of advanced MTC. Vandetanib treatment has resulted in objective responses in a significant fraction of MTC patients and has extended progression-free survival (5). However, no complete responses have been reported, and many MTC patients progress despite vandetanib or other kinase inhibitors. Clearly new molecular targets and therapies for MTC are urgently needed.

Other than *RET* mutations, few molecular abnormalities have been elucidated in either MEN 2-related or sporadic MTC (6–9) (http://www.sanger.ac.uk/genetics/CGP/cosmic/), although recent reports have documented frequent activating mutations in *HRAS* and *KRAS* in the subset of sporadic MTC with wild-type *RET* (10–12). Interestingly, common abnormalities found in other cancers, such as *TP53*, *RB1*, *PIK3CA*, and *BRAF* gene mutations, are extremely uncommon or absent in MTC (13–16).

There is also a gap in our understanding of key signaling pathways involved in *RET* wild-type and mutant tumors, both in hereditary and sporadic cases. To begin to shed light on the biology of MTC and to attempt to identify new molecular targets for therapy, we sequenced the coding exons of approximately 21,000 genes in 17 cases of sporadic MTC.

## Patients and Methods

### Preparation of clinical samples

For the discovery screen of 17 samples for whole-exome sequencing, fresh-frozen tumor from clinically annotated sporadic MTCs and matched blood were obtained from patients under an institutional review board-approved protocol at the Johns Hopkins Hospital. Tumor tissue was analyzed by frozen section to assess neoplastic cellularity. Tumors were macrodissected to remove residual normal tissue and enhance neoplastic cellularity, as confirmed by multiple frozen sections. Estimated neoplastic cellularity was approximately 80%. For the prevalence screen of 40 samples for Sanger sequencing of candidate genes, tumor DNA from clinically annotated sporadic and hereditary MTCs and matched normal DNA were obtained from patients treated under institutional review board protocols from Johns Hopkins Hospital, Memorial Sloan Kettering Cancer Center, University of Sao Paulo School of Medicine, University of Halle-Wittenberg, and University of Sydney.

### Preparation of Illumina genomic DNA libraries

Genomic DNA libraries were prepared following Illumina's (Illumina, San Diego, CA) suggested protocol with the following modifications (1). Three micrograms of genomic DNA from tumor or normal cells in 100 μL of Tris/EDTA buffer were fragmented in a Covaris sonicator (Covaris, Woburn, MA) to a size of 100–500 bp. DNA was purified with a PCR purification kit (catalog no. 28104; QIAGEN, Valencia, CA) and eluted in 35 μL of elution buffer included in the kit (2). Purified, fragmented DNA was mixed with 40 μL of $H_2O$, 10 μL of 10 × T4 ligase buffer with 10 mM ATP, 4 μL of 10 mM deoxyribonucleotide triphosphate (dNTP), 5 μL of T4 DNA polymerase, 1 μL of Klenow polymerase, and 5 μL of T4 polynucleotide kinase. All reagents used for this step and those described below were from New England Biolabs (Ipswich, MA) unless otherwise specified. The 100-μL end-repair mixture was incubated at 20°C for 30 minutes, purified by a PCR purification kit (catalog no. 28104; QIAGEN), and eluted with 32 μL of elution buffer (EB) (3). To A-tail, all 32 μL of end-repaired DNA was mixed with 5 μL of 10× buffer (New England Biolabs buffer 2), 10 μL of 1 mM dATP, and 3 μL of Klenow (exo-). The 50-μL mixture was incubated at 37°C for 30 minutes before DNA was purified with a MinElute PCR purification kit (catalog no. 28004; QIAGEN). Purified DNA was eluted with 12.5 μL of 70°C EB and obtained with 10 μL of EB (4). For adaptor ligation, 10 μL of A-tailed DNA was mixed with 10 μL of PE-adaptor (Illumina), 25 μL of

2× rapid ligase buffer, and 5 μL of rapid ligase. The ligation mixture was incubated at room temperature or 20°C for 15 minutes (5). To purify adaptor-ligated DNA, 50 μL of ligation mixture from step (4) was mixed with 200 μL of buffer NT from a NucleoSpin Extract II kit (catalog no. 636972; CLONTECH, Mountain View, CA) and loaded into a NucleoSpin column. The column was centrifuged at 14000 × *g* in a desktop centrifuge for 1 minute, washed once with 600 μL of wash buffer (NT3 from CLONTECH), and centrifuged again for 2 minutes to dry completely. DNA was eluted in 50 μL elution buffer included in the kit (6). To obtain an amplified library, 10 PCRs of 25 μL each were set up, each including 13.25 μL of $H_2O$, 5 μL of 5× Phusion HF buffer, 0.5 μl of a dNTP mix containing 10 mM of each dNTP, 0.5 μL of Illumina PE primer no. 1, 0.5 μL of Illumina PE primer no. 2, 0.25 μL of Hotstart Phusion polymerase, and 5 μL of the DNA from step 5. The PCR program used was as follows: 98°C 1 minute; 6 cycles of 98°C for 20 seconds, 65°C for 30 seconds, and 72°C for 30 seconds; and 72°C for 5 minutes. To purify the PCR product, 250 μL PCR mixture (from the 10 PCRs) was mixed with 500 μL NT buffer from a NucleoSpin Extract II kit and purified as described in step 5. Library DNA was eluted with 70°C elution buffer and the DNA concentration was estimated by absorption at 260 nm.

## Exome and targeted subgenomic DNA capture

Human exome capture was performed following a protocol from Agilent's SureSelect paired-end version 2.0 human exome kit (Agilent, Santa Clara, CA) with the following modifications: (1) a hybridization mixture was prepared containing 25 μl of SureSelect Hyb no. 1, 1 μL of SureSelect Hyb no. 2, 10 μL of SureSelect Hyb no. 3, and 13 μL of SureSelect Hyb no. 4; (2) 3.4 μL (0.5 μg) of the PE-library DNA described above, 2.5 μL of SureSelect Block no. 1, 2.5 μL of SureSelect Block no. 2, and 0.6 μL of SureSelect Block no. 3 were loaded into 1 well in a 384-well Diamond PCR plate (catalog no. AB-1111; Thermo-Scientific, Lafayette, CO), sealed with microAmp clear adhesive film (catalog no. 4306311; Applied Biosystems Inc, Carlsbad, CA) and placed in GeneAmp PCR system 9700 thermocycler (Life Sciences Inc, Carlsbad CA) for 5 minutes at 95°C and then held at 65°C (with the heated lid on); (3) 25–30 μL of hybridization buffer from step 1 was heated for at least 5 minutes at 65°C in another sealed plate with the heated lid on; (4) 5 μL of SureSelect Oligo Capture Library, 1 μL of nuclease-free water, and 1 μL of diluted ribonuclease block (prepared by diluting ribonuclease block 1:1 with nuclease free water) were mixed and heated at 65°C for 2 minutes in another sealed 384-well plate; (5) while keeping all reactions at 65°C, 13 μL of hybridization buffer from step (3) was added to the 7 μL of the SureSelect Capture Library Mix from step 4 and then the entire contents (9 μL) of the library from step 2. The mixture was slowly pipetted up and down 8–10 times; and (6) the 384-well plate was sealed tightly, and the hybridization mixture was incubated for 24 hours at 65°C with a heated lid.

After hybridization, 5 steps were performed to recover and amplify captured DNA library: (1) magnetic beads for recovering captured DNA: 50 μl of Dynal MyOne Streptavidin C1 magnetic beads (catalog no. 650.02; Invitrogen Dynal, AS Oslo, Norway) was placed in a 1.5-mL microfuge tube and vigorously resuspended on a vortex mixer. Beads were washed 3 times by adding 200 μL of SureSelect Binding buffer, mixing on a vortex for 5 seconds, and then removing the supernatant after placing the tubes in a Dynal magnetic separator, and after the third wash, beads were resuspended in 200 μL of SureSelect Binding buffer; (2) to bind captured DNA, and the entire hybridization mixture described above (29 μL) was transferred directly from the thermocycler to the bead solution and mixed gently; the hybridization mix/bead solution was incubated in an Eppendorf thermomixer at 850 rpm for 30 minutes at room temperature; (3) to wash the beads, the supernatant was removed from beads after applying a Dynal magnetic separator, and the beads were resuspended in 500 μL SureSelect wash buffer no. 1 by mixing on vortex mixer for 5 seconds and incubated for 15 minutes at room temperature, and wash buffer no. 1 was then removed from the beads after magnetic separation, and the beads were further washed 3 times, each with 500 μL prewarmed SureSelect wash buffer no. 2 after incubation at 65°C for 10 minutes; after the final wash, SureSelect wash buffer no. 2 was completely removed; (4) to elute captured DNA, the beads were suspended in 50 μl SureSelect EB, vortex mixed, and incubated for 10 minutes at room temperature. The supernatant was removed after magnetic separation, collected in a new 1.5-mL microcentrifuge tube, and mixed with 50 μL of SureSelect neutralization buffer, and DNA was purified with a QIAGEN MinElute column and eluted in 17 μL of 70°C EB to obtain 15 μL of captured DNA library; and (5) the captured DNA library was amplified in the following way: 15 PCRs, each containing 9.5 μL of $H_2O$, 3 μL of 5× Phusion HF buffer, 0.3 μL of 10 mM dNTP, 0.75 μL of dimethylsulfoxide, 0.15 μL of Illumina PE primer no. 1, 0.15μL of Illumina PE primer no. 2, 0.15 μL of Hotstart Phusion polymerase, and 1 μL of captured exome library, were set up. The PCR program used was: 98°C for 30 seconds; 14 cycles of 98°C for 10 seconds, 65°C for 30 seconds, 72°C for 30 seconds, and 72°C for 5 minutes. To purify PCR products, 225 μL PCR mixture (from 15 PCRs) was mixed with 450 μl NT buffer from the NucleoSpin Extract II kit and purified as described above. The final library DNA was eluted with 30 μL of 70°C EB, and DNA concentration was estimated by OD 260 measurement.

## Somatic mutation identification by massively parallel sequencing

Captured DNA libraries were sequenced with the Illumina GAIIx genome analyzer. Sequencing reads were analyzed and aligned to human genome hg18 with the Eland algorithm in CASAVA 1.6 software (Illumina). A mismatched base was identified as a mutation only when the following occurred: (1) it was identified by 5 or more distinct pairs; (2) the number of distinct tags containing a particular mismatched base was at least 10% of the total distinct tags; and (3) it was not present in greater than 0.5% of the tags in the matched normal sample. The single-nucleotide polymorphism search databases included the http://www.ncbi.nlm.nih.gov/projects/SNP/.

## Evaluation of genes in additional tumors and matched normal controls

Based on discovery screen findings, a prevalence screen was carried out for the *RET*, *HRAS*, *KRAS*, *MDC1*, *PTPRJ*, *SF3B1*, and *SF3B3* genes. The entire coding region of these genes was sequenced in an additional 40 MTC and matched normal specimens. PCR amplification and Sanger sequencing were performed following protocols described previously using the primers listed in Supplemental Table 1 (17), published on

The Endocrine Society's Journals Online web site at http://jcem.endojournals.org.

## Results

### Discovery exomic sequencing of MTC

DNA was purified from 17 sporadic MTCs as well as matched nonneoplastic tissues and used to generate 34 libraries suitable for massively parallel sequencing. After capture of the coding sequences with a SureSelect paired-end version 2.0 human exome kit (Agilent), the DNA was sequenced using an Illumina GAIIx instrument. The enrichment system included 50 Mb of protein-coding exons from the human genome, corresponding to approximately 21,000 genes.

The average distinct coverage of each base in the targeted region was approximately 94-fold, and 90.4% of targeted bases were represented by at least 10 reads. Using stringent criteria for the analysis of these data (see *Patients and Methods*), we identified 305 high-confidence somatic mutations in 283 genes (Supplemental Table 2). To confirm the specificity of our mutation-calling criteria, we evaluated 225 of the 305 candidate mutations by Sanger sequencing and confirmed 196 of them (87%); 9 mutations (4%) could not be amplified by PCR because of an unusually high guanine-cytosine content, difficulty in the design of unique primers, or other unknown factors preventing specific amplification and sequencing of the locus; and the remaining 20 mutations (9%) were not present at levels detectable by Sanger sequencing. The number of high-confidence somatic mutations per tumor averaged 17.9 (range from 4 to 29 and SD ± 8.8) (Table 1). Of the 305 high-confidence somatic changes, there were 248 single base pair substitutions, 1 2-bp substitution,18 nonsense mutations, and 16 essential splice site mutations as well as 22 indels. The most common base pair substitutions in MTCs were C:G>T:A (27.2%) and A:T>G:C (18%) transitions (Supplemental Table 3).

In sporadic MTCs, the most commonly mutated gene was *RET*, which was somatically mutated at a prevalence of 71% in the discovery screen. Several of the other somatically mutated genes appeared to cluster in specific functional classes, including spliceosome and DNA repair. Of particular interest, we found 2 mutations in *SF3B1* and 1 mutation in *SF3B3*, which have recently been shown to be mutated in chronic lymphocytic leukemia (18–20).

In all 5 MTC samples with wild-type *RET*, *HRAS*, and *KRAS*, we found no recurrent mutated genes, although 2 *RET*- and *RAS*-negative MTCs had a *MDC1* or *ATM* somatic mutation, suggesting a potential role for the DNA damage pathway in *RET*-negative MTC. These findings suggest that there may be no other predominant driver mutations in the tumor subset without *RET* and *RAS* mutations. Interestingly, the tumors without *RET*, *HRAS*, or *KRAS* mutations were found to have significantly fewer mutations overall (8.4 vs 21.9, $P < .001$ by 2 tailed *t* test).

**Table 1.** Summary of Genomic Analysis of MTC

| | Discovery | Validation | Total |
|---|---|---|---|
| Samples | 17 | 40 | 57 |
| RET | 12 | 31 | 43 |
| HRAS | 0 | 7 | 7 |
| KRAS | 0 | 2 | 2 |
| MDC1 | 2 | 1 | 3 |
| Sporadic MTC | 17 | 19 | 36 |
| High-confidence mutations | 305 | | |
| High-confidence mutations/tumor | 17.9 | | |

### Validation of the most commonly mutated genes

Other than *RET*, the genes that were mutated in at least 2 of the 17 MTCs were *MDC1*, *SF3B1*, *MGAM*, *DOCK9*, *SEMA6A*, *TDG*, and *DISP2* (Supplemental Table 2). To evaluate the incidence of mutations in these closely related genes, as well as genes previously implicated in MTC tumorigenesis, we analyzed the sequences of *RET*, *HRAS*, *KRAS*, *MDC1*, *PTPRJ*, *SF3B1*, and *SF3B3* in 40 additional MTCs, comprised of 19 sporadic and 21 hereditary MTCs and their corresponding normal tissues. *PTPRJ* was included in the validation screen for its potential biological importance as a membrane-associated phosphatase known to regulate the activity of *RET* (21), although only a single example of mutation was seen in the discovery screen. *MGAM*, *DOCK9*, and *SEMA6A* were not included in this validation screen because their mutations were determined not to significantly alter the gene product. Three of the 4 *TDG* mutations and 1 of the 2 *DISP2* were not confirmed on Sanger sequencing and were also not included in the validation screen.

In the additional 40 sporadic and hereditary MTCs, somatic mutations of *RET, HRAS, KRAS,* and *MDC1* were identified in 78%, 18%, 5%, and 3%, respectively (Supplemental Tables 1 and 4). *HRAS* and *KRAS* mutations occurred in previously described mutational hot spots: codons 61 and 117 and codon 12, respectively (9). No pathogenic mutations in *RET* were detected outside exons 8, 10, 11, and 13–16. The *RET*, *HRAS*, and *KRAS* mutations were mutually exclusive. Collectively, in the discovery and validation screens, 91% of all tumors harbored *RET*, *HRAS*, or *KRAS* mutations.

*MDC1* (mediator of DNA damage checkpoint protein 1) is a key component of the DNA damage response, binding to γ-H2AX at DNA double-strand breaks, and participating in the recruitment of key factors including *ATM*

(ataxia telangiectasia mutated), *BRCA1*, and *TP53*. Of significant interest, all 3 *MDC1* mutations observed in this study clustered in the C-terminal BRCA1 binding domain, including a nonsense mutation at Q1834 that truncates this domain. *MDC1* loss of function could produce abnormalities in both homologous recombination and nonhomologous end joint repair pathways (22). Importantly, tumors with *MDC1* and *ATM* mutations have the potential of being more radiosensitive (23).

The remaining genes (*PTPRJ*, *SF3B1*, and *SF3B3*) were not mutated in any of the additional 40 tumors analyzed.

## Discussion

In addition to confirming the dominant role of *RET* in MTC pathogenesis, our data confirm the critical role of *RAS* in *RET* mutation-negative tumors. The *RET*, *KRAS*, and *HRAS* mutations observed in MTC were mutually exclusive and collectively found in the vast majority of the tumors (Table 1). *RET*, a receptor tyrosine kinase, acts in part through activation of RAS signaling. Thus, remarkably, MTC is characterized by unusually intense oncogene predominance, with almost universal activation of mutationally dysregulated RAS pathway signaling. These findings, indicating the central importance of the RAS pathway in MTC, have potential therapeutic implications and support the hypothesis that inhibition of the RAS pathway may be an effective strategy for treating MTC.

However, experience with another tumor type with *RAS* oncogene predominance, pancreatic ductal adenocarcinoma (PDAC), may decrease this optimism. In PDACs, *KRAS* mutations occur early and very frequently (24–28). Clinical targeting of RAS in PDAC has been disappointing (29, 30). However, PDAC also harbors recurrent mutations in several other, RAS-unrelated genes (24), and these genes may provide alternative survival pathways for PDAC. In contrast, MTC harbors no other recurrently mutated genes, and, by analogy to chronic myelocytic leukemia, may be especially sensitive to effective targeting of RAS and its effector pathways. In this regard, it is interesting that the average number of mutations in MTC, 17.9, is lower than that detected in most other solid tumors assessed via a similar screening strategy (24, 31–36), further suggesting the potential reliance of MTC on the RAS signaling pathway.

The especially low number of mutations in MTC, especially in cases without *RET* or *RAS* mutations, is noteworthy. In a recent study of low-grade serous ovarian carcinoma (37), a similar paucity of mutations was found. Based on the low numbers of mutations per tumor, it was concluded that the normal precursor cells are likely to replicate slowly and that there were few bottlenecks to subsequent tumor development. Indeed, apart from MEN 2 in which precancerous C-cell hyperplasia stems from germline RET gene mutations, there is scant pathological or molecular evidence for multistage tumorigenesis in MTC. An early estimate of the number of hits required for MTC tumorigenesis suggested that few such events were required (38). The difference in the number of mutations between MTC tumors with *RET* or *RAS* mutations, and those without, further suggests that there may be fundamental differences in the tumorigenic pathways used. In addition, one may speculate that a relatively high fraction of the mutations in the tumors without *RET* and *RAS* mutations are drivers. Potentially, larger-scale DNA changes, translocations, epigenetic changes, or alterations in noncoding RNA could be important.

It will be important to determine whether the existence of a *RAS* mutation influences the response to targeted therapy in MTC. As mentioned above, the RET inhibitor vandetanib can extend progression-free survival in patients with MTC (5). However, it is not yet clear whether this clinical benefit extends to MTC patients with *RAS* mutations. In subgroup analyses, Wells et al (5) showed that patients confirmed to have *RET* M918T mutation-negative tumors had slightly reduced response rates and progression-free survival compared with M918T mutation-positive patients. In colorectal and non-small-cell lung cancer patients, treatment with antiepithelial growth factor receptor therapy is ineffective in tumors with *KRAS* mutations (39, 40). If there proves to be a difference in the response of tumors to treatment with *RET* inhibitors based on a combination of *RAS* and *RET* mutation status, the evaluation of mutations in these two genes may become an important parameter for patient stratification.

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