Association of the Genomic Profile of Medullary Thyroid Carcinoma with Tumor Characteristics and Clinical Outcomes in an International Multicenter Study

Molecular Profile of Medullary Thyroid Carcinoma (MTC) and Its Impact on Tumor Characteristics and Clinical Outcome: An International Multicentric Study of 290 Patients

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Running title: Molecular alterations in medullary thyroid carcinoma.

Abstract

Background: The prognostic impact of germline *RET*, somatic *RET* and *RAS* mutations and their relationship to clinicopathologic parameters and outcomes is poorly defined in medullary thyroid carcinoma (MTC) and needs to be clarified.

Design: Molecular profile, including germline and somatic *RET* and *RAS* mutations were detected using various molecular platforms in 290 primary MTCs from 6 tertiary centers, including 239 from prior an international MTC grading system (IMTCGS) cohort and 51 new cases.

Results: *RET* germline mutations were detected in 40 patients (16.3%). Somatic *RET* and *RAS* mutations were seen in 136 (46.6%) and 57 (19.8%) respectively. *RET* M918T was the most common somatic *RET* mutation (n=75).

Compared with wild type (WT) MTCs, those with *RET* germline mutation were associated with younger age at presentation. MTCs with *RET* somatic mutations were associated with female sex, larger tumor size, advanced AJCC prognostic group, vascular invasion, and high IMTCGS grade. There was no significant difference between MTCs with somatic *RAS* mutations and WT MTCs. When compared with other *RET* somatic mutations, *RET* M918T was more commonly associated with younger age, AJCC prognostic group 4, vascular invasion, extrathyroidal extension, and positive margin.

The presence of any *RET* mutation, *RET* M918T, and *RAS* mutations did not significantly impact overall and disease specific survival (OS and DSS). RET somatic or germline mutations were significant adverse prognostic factor for distant metastasis free survival (DMFS) on univariate survival analysis but lost its significance on multivariate analysis when adjusted for grade and stage. There were no outcome differences between *RET* somatic and *RET* germline mutations, or between *RET* M918T and other *RET* mutations.

Other recurrent molecular alterations included *TP53* (4.2%), *ARID2* (2.9%), *SETD2* (2.9%), *KMT2A* (2.9%), and *KMT2C* (2.9%), *PIK3CA* (1.0%), *ATM* (2.3%), *VHL* (1.0%) and *TERT* promoter mutation (1.0%). Among them, *TP53* mutations were associated with decreased OS, DSS, and LRRFS on univariate survival analysis. There was no association between *TP53* mutations and grade or AJCC prognostic group.

Conclusions: *RET* somatic mutations correlate with high-grade, aggressive primary tumor characteristics, and decreased DMFS. *RET* M918T is associated with aggressive primary tumors but does not impact outcome. We further identified *TP53* mutation as an adverse molecular signature to predict decreased OS, DSS, and LRRFS in MTCs.

Introduction

Medullary thyroid carcinoma (MTC), a neuroendocrine carcinoma derived from parafollicular c-cells, accounts for approximately 2% of all thyroid malignancy and 8% of thyroid cancer-related mortality [1]. It may occur sporadically or in the setting of a germline mutation of *RET* protooncogene (i.e. multiple neuroendocrine neoplasia type 2, MEN2) [2-5]. A large proportion of sporadic MTCs harbor *RET* (particularly *RET* M918T) or *RAS* somatic mutations [6-10], making them potential candidates for kinase inhibitor targeted therapies [11].

In 2022, we established an international MTC consortium to develop and validate a powerful prognosticator of MTC, namely the International Medullary Thyroid Carcinoma Grading System (IMTCGS) based on mitotic index, Ki67 proliferation index, and/or tumor necrosis [2]. Although data for *RET* germline mutations were collected, the underlying molecular profile and its correlation with clinicopathologic features and outcome of MTC remained to be elucidated.

In this study, we investigated the molecular signatures of a large multicentric cohort of 290 patients with primarily resected MTC using RT-PCR based platforms and six different next generation sequencing (NGS) platforms. The aims are two-fold: first to clarify the prognostic significance of somatic *RET* or *RAS* mutations and their correlations with various clinicopathologic parameters, including IMTCGS grade; and second to identify other molecular alterations in MTCs and their prognostic roles.

Materials and methods

Study cohort

This retrospective cohort study included 290 resected primary MTCs from six tertiary centers (University of Bologna Medical Center [UB], Bologna, Italy: n=64; Memorial Sloan Kettering Cancer Center [MSKCC], New York, NY, USA: n=54; Institut Gustave Roussy, Villejuif, France: n=45; Brigham and Women's Hospital [BWH], Boston, MA, USA: n=44; Royal North Shore Hospital, Sydney, Australia: n=42, and Emory University Hospital Midtown [EU], Atlanta, GA, USA: n=41). Among them, 239 patients were included in the prior IMTCGS cohort [2], whereas the remaining 51 were new patients (UB: n=6; MSKCC: n=4; and EU n=41). One center (BWH) included only patients with somatic MTC, whereas the cases from other centers also contained patients with germline *RET* mutations.

Molecular platforms

Somatic molecular alterations, including *RET* and *RAS* mutations, were detected using either RT-PCR-based platforms targeting only *RET* and/or *RAS* genes (n=99) or six NGS platforms (n=191). Details of the NGS platforms utilized were either commercially available or were described previously [12-17] (briefly summarized in **Supplementary Table 1**). Number of cases tested using each NGS platform was as follows: a custom-designed multi-gene panel (n=104), MSK-IMPACT (n=54), ion Ampliseq™ cancer hotspot v. 2 (CHP2, Thermofisher Scientific, n=17), OncoPanel (n=14), Paradigm Cancer Diagnostic (PCDx) platform (Paradigm diagnostics, n=1), and CARIS NGS platform (CARIS life sciences, n=1).

Clinicopathologic review, outcome, and statistical analysis

Clinicopathologic review were performed at each individual participating site. Outcome data, including overall survival (OS), disease specific survival (DSS), distant metastasis free survival (DMFS), and locoregional recurrence free survival (LRRFS), were collected. Statistical analyses were performed using the SPSS software 24.0 (IBM Corporation, Armonk, NY, U.S.). Univariate survival analysis was performed using log rank test for categorical variables and Cox proportional hazards model for continuous variables. Multivariate survival analysis using Cox proportional hazards model adjusted for grade and stage was subsequently conducted. Additionally, comparisons of the clinicopathologic features among each molecular subgroup (*RET* germline mutations, *RET* somatic mutations, *RAS* somatic mutations, and *RET/RAS* wild type) were performed using Chi square test or Fisher's exact test for categorical variables and two-tailed Student's t test for continuous variables.

Results

Clinicopathologic characteristics of the study cohort

This retrospective study cohort had 290 patients with resected primary thyroid MTC. The median age of presentation was 57 years (range: 7 – 88 years, **Table 1**). The male to female ratio was 1:1.1. AJCC prognostic group 4, vascular invasion, extrathyroidal extension, positive margin, and IMTCGS high grade were identified in 39.3%, 42.1%, 28.8%, 18.6%, and 24.8% of the cases respectively. Twenty-one patients (8.2%) had distant metastasis at presentation. External beam radiation therapy and kinase inhibitors were given to 8.2% and 8.7% of patients respectively. The kinase inhibitors used

included selpercatinib (n=7), vandetanib (n=5), cabozantinib (n=3), pralsetinib (n=1), sorafenib (n=1), LOXO-292 (n=1), RAD001 (n=1), and RAD100 (n=1).

RET germline mutations

After excluded the BWH subgroup which contained only somatic MTCs, the frequency of RET germline mutations was 16.3% (40/246, **Figure 1 and Supplementary Table 2**). The most frequently detected germline mutations were C609Y (n=14), C634R (n=8), and G533C (n=3). Among these familial MTCs, *RET* or *RAS* somatic mutations were detected in three cases: one case had germline *RET* Y791F and somatic R*ET* M918T, one had germline *RET* V804Y and somatic *RET* M918T, and the third had *RET* C634R germline mutation and *HRAS* G13R somatic mutation.

RET somatic mutations

RET somatic mutations were detected in 136 cases of MTC (46.6% overall, 53.6% in sporadic MTCs, **Table 1**, **Figure 1**, and **Supplementary Table 1**). Among them, *RET* M918T was the most common somatic mutations, being detected in 75 cases. Other common *RET* somatic mutations affected codons 634 (n=16), 630 (n=11), 620 (n=7), and 883 (n=6).

RAS somatic mutations

RAS somatic mutations were examined in 288 cases, and were identified in 57 MTCs (19.8% in the entire cohort, 22.4% in sporadic MTCs, 44.9% in *RET*-WT sporadic MTCs), including *HRAS* mutations in 37 cases (Q61R n=21, G13R n=10, Q61K n=3, Q61L n=2, and G13V n=1), *KRAS* mutations in 20 cases (G12R n=8, G12V n=3, Q61R n=3, Q61L n=1, Q61K n=1, D54N n=1, A18D n=1, P34L n=1, and C186Mfs*16 n=1), and *NRAS* Q61K mutation in 1 case. One MTC harbored two *RAS* somatic mutations, being *HRAS* G13R and *KRAS* C186Mfs*16. *RAS* and *RET* somatic mutations were mutually exclusive.

In our cohort, 61 MTCs (21.0%) were devoid of *RET* (somatic or germline) or *RAS* mutations and were grouped as *RET/RAS* wild type (WT).

Correlation of *RET/RAS* mutations with clinicopathologic parameters

The clinicopathologic characteristics according to R*ET* and *RAS* mutations status is shown in **Table 1**.

Compared with WT MTCs, MTC occurring in the familial setting was associated with younger age at presentation (median age: 42 Years in *RET* germline mutation group, 58 years in WT). Other clinicopathologic characteristics did not differ between the two groups.

MTCs with *RET* somatic mutations were associated with aggressive tumor characteristics at presentation compared with WT MTC, including larger tumor size (median size: 2.1 cm in *RET* somatic mutations, 1.9 cm in WT), AJCC prognostic group 4 (56.6% in *RET* somatic mutations, 24.6% in WT), vascular invasion (55.9% in *RET* somatic mutations, 32.8% in WT), and high IMTCGS grade (35.3% in *RET* somatic mutations, 18.0% in WT). Additionally, patients with *RET* somatic mutations were more commonly male (male to female ratio: 1:0.7 in *RET* somatic mutation group, 1:1.4 in WT) and more commonly treated with kinase inhibitor therapy (15.2% in *RET* somatic mutation group, 3.3% in WT group).

Similarly, compared with MTCs with *RET* germline mutations, MTC with *RET* somatic mutations were more aggressive characterized by larger tumor size, AJCC prognostic group 4, vascular invasion, tumor necrosis, high mitotic index, high Ki67 proliferation rate, and distant metastasis at presentation.

The clinicopathologic features did not differ significantly between MTCs with somatic *RAS* mutations and WT MTCs.

RET M918T somatic mutation was considered a high-risk mutation in this study. When compared with other *RET* somatic mutations, *RET* M918T was more commonly associated with younger age (median age: 52 years in *RET* M918T, 63 years in other *RET* somatic mutations), AJCC prognostic group 4 (69.3% in *RET* M918T, 40.0% in other *RET* somatic mutations), vascular invasion (65.3% in *RET* M918T, 43.3% in other *RET* somatic mutations), extrathyroidal extension (46.7% in *RET* M918T, 20.0% in other *RET* somatic mutations), and positive surgical margin (30.7% in *RET* M918T, 15.0% in other *RET* somatic mutations**, Supplementary Table 3**). Other clinicopathologic parameters did not differ between MTCs with *RET* M918T and those with other *RET* somatic mutations.

The impacts of *RET* and *RAS* mutations on clinical outcomes

The prognostic impact of *RET* and *RAS* mutations were determined using univariate survival analysis by log rank test and the results are shown in **Table 2** and **Figure 1**. In brief, *RET* or *RAS* mutation status did not impact OS and DSS.

Compared with WT MTCs, those with *RET* somatic or *RET* germline mutations were associated with shortened DMFS ($p<0.05$). The 10-year DMFS in MTCs with *RET* somatic mutations, MTCs with *RET* germline mutations, and WT MTCs was 49%, 47%, and 75% respectively. There was also a non-significant trend for *RAS*-mutated MTCs to be associated with improved DMFS (10-year DMFS in *RAS*-mutated MTCs: 88%, $p=0.081$).

RET or *RAS* mutation profile failed to reach significant levels on multivariate survival analysis using Cox proportional hazards model adjusted for IMTCGS grade and AJCC prognostic group (**Supplementary Table 4**, $p>0.05$). IMTCGS grade and AJCC prognostic group remained independent adverse prognostic factors (IMTCGS grade: hazard ratio 2.031, 95% confidence interval 1.230 – 3.353, $p=0.006$; AJCC stage group 4: hazard ratio 1.478, 95% confidence interval 1.221 – 1.789, $p<0.001$).

Other molecular alterations in MTC

Other mutations were examined in 191 cases using six different NGS platforms ranging from 26 genes to whole exome sequencing. The detailed mutation profile is provided in **Supplementary Table 5**. Recurrent somatic mutations or fusions detected included *TP53* (8/191, 4.2%), *PIK3CA* (2/191, 1.0%), *VHL* (2/191, 1.0%). *TERT* promoter mutation (2/191, 1.0%), *ATM* (2/87, 2.3%), *ARID2* (2/70, 2.9%), *SETD2* (2/70, 2.9%), *KMT2A* (2/70, 2.9%), and *KMT2C* (2/70, 2.9%). The presence of TP53 mutations was associated with decreased OS (log rank test, $p=0.027$, **Supplementary Figure 1**), DSS ($p=0.008$), and LRRFS ($p=0.018$), and showed a non-significant trend towards shortened DMFS ($p=0.074$). There was no correlation between TP53 mutations and IMTCGS grade or AJCC prognostic group ($p=1.000$). Other molecular alterations did not significantly correlate with clinicopathologic characteristics or survival.

Discussion

In this multicentric retrospective study, we presented a complete dataset including molecular signatures, detailed clinicopathologic features, and outcomes of 290 patients, the largest MTC cohort to date. Our key findings were 1) *RET* somatic mutations were associated with aggressive tumor characteristics, IMTCGS high grade, and decreased DMFS. However, the prognostic values of *RET* somatic mutations were not independent of grade and stage; 2) MTCs with *RET* M918T somatic mutations showed tumor aggressiveness in the primary resection (such as AJCC prognostic group 4, vascular invasion, extrathyroidal extension, and positive margin), but did not impact survival; and 3) *TP53* mutation was identified as a novel adverse molecular signature for survival affecting 4.2% of MTC.

Germline *RET* mutations were detected in 16.3% of the study cohort, lower than the ~25% rate of familial cases reported in the literature [18]. The relatively low frequency of germline *RET* mutations may be explained by the selection bias of our study cohort towards adult patients: only 4 patients (1.4%) of our cohort were age 18 years or under. As the current American Thyroid Association (ATA) guideline recommended prophylactic thyroidectomy at young age for patients with hereditary MTCs, such patients were likely not included in our cohort giving the selection bias towards adult patients [18]. Consistent with what were previously reported [5,19-21], we found that *RET* germline mutations affected codons 533, 609, 611, 618, 634, 791, 804, and 918, with codon 618 being the most prevalent, and that hereditary MTCs occurred at a younger age compared with sporadic MTCs.

RET somatic mutations have been identified in 45% to 70% of sporadic MTCS and have been shown to be associated with larger tumor size [22], high T stage [23], nodal metastasis [22-24], distant metastasis [22-24], advanced stage [22-24], and decreased survival (overall and disease-free) [22-24] in MTCs. Similarly, we have shown here that MTCs with *RET* somatic mutations had larger tumor size, advance AJCC prognostic group, and decreased DMFS. However, the prognostic values of RET somatic mutations were lost on multivariate survival analysis when adjusted for IMTCGS grade and AJCC prognostic group, while grade and stage remained independent prognostic factors. Together, these data imply that *RET* somatic mutation was associated with more aggressive tumor characteristics and adverse outcome, but its role in MTC may not be independent of grade and stage. In a study of 100 sporadic MTCs, Elisei et al. identified *RET* somatic mutation and advance stage as the only two factors independently correlated with persistent disease [23]. There were several differences between their study and the current study. First, Elisei et al. included only sporadic MTCs, whereas our cohort contained both hereditary and sporadic cases. Second, *RAS* somatic mutation was not studied in Elisei et al., therefore, their *RET* WT group would include MTCs with *RAS* mutations which had a trend towards better prognosis. Third, Elisei et al only performed correlation analysis between persistent disease and mutation/stage using multivariable logistic regression model. Multivariant survival analysis was not done. The fact that driver mutations are not independent from stage and other clinico-pathologic factors such as grade is not unique to MTC. Indeed, the same scenario applies to follicular cell derived thyroid carcinomas where *BRAF* V600E mutation was shown to be not independent from clinico-pathologic features such as stage in predicting mortality [25].

Additionally, we have shown for the first time that *RET* somatic mutation correlate with IMTCGS high grade. Najdawi et al. previously reported that there was no correlation between *RET* somatic mutation and IMTGS grade in 44 sporadic MTCs [26], These 44 patients were also included in the current study. We were able to establish an association between IMTCGS high grade and *RET* somatic mutations in the current study, possibly for two reasons: first, we expanded the cohort size to 290 cases; and second, we excluded cases with *RAS* somatic mutations from the control (WT) group.

It is worthwhile to mention that not all IMTCGS high grade MTCs harbored *RET* somatic or germline mutations. Among the 72 cases of high grade MTCs, 55 (76.4%) had *RET* somatic or germline mutations, 9 (12.5%) had *RAS* mutations, whereas the remaining 11 did not harbor *RET* or *RAS* mutations. Therefore, not all patients with IMTCGS high grade MTCs are candidate for kinase inhibitor therapies.

RET M918T somatic mutation, the most prevalent *RET* somatic mutation in MTC, has been implied in several studies to be an adverse molecular signature. Schilling et al. [27] showed that *RET* M918T was associated with decreased OS and increased risk of distant metastasis in 34 patients with sporadic MTCs. However, the authors only examined *RET* M918T mutations in their study, and their control group theoretically included both MTCS with other *RET* somatic mutations and MTCs without *RET* mutations. Therefore, it was impossible to directly compare *RET* M918T somatic mutations with other *RET* somatic mutations in this study. Romei et al. [28] showed that *RET* M918T mutations was associated with larger tumor size and was relatively infrequent in MTC < 1 cm in size. Moura et al. found that MTCs with *RET* somatic mutations involving exons 15 and 16 (including 87% of M918T and 13% of A883F) had higher prevalence of nodal metastasis, stage IV disease, and persistent disease compared with MTCs with other *RET* somatic mutations in 52 sporadic MTCs [29]. In contrast, Najdawi et al. [26] showed that *RET* somatic mutations affecting exons 15 and 16 did not impact DSS and progression free survival and did not correlate with IMTCGS grade. In the current study including 75 cases with *RET* M918T somatic mutations and 60 cases with other *RET* somatic mutations, we found that *RET* M918T somatic mutations were associated with younger age, advanced AJCC prognostic group, and other aggressive tumor characteristics (e.g. vascular invasion, extrathyroidal extension, and positive margin) compared with MTCs with other *RET* somatic mutations. However, *RET* M918T somatic mutations lacked any prognostic values to predict OS, DSS, DMFS, and LRRFS in MTCs.

RAS mutations are the predominant driver mutations in *RET*-WT sporadic MTC, being detected in 11% (range: 9% to 20%) of MTC overall, 13% (range: 0% to 43%) of sporadic MTCs, and 61% (range: 0% to 81%) of *RET*-WT sporadic MTCs [7,9,10,30-33]. Similarly, we detected *RAS* mutations in 19.8% of all MTCs, 22.4% of sporadic MTCs, and 44.9% of *RET*-WT sporadic MTCs. Although we showed a non-significant trend of *RAS*-mutated MTCs with improved DMFS, overall RAS mutations did not impact tumor characteristics or outcomes in MTC as shown previously [24].

It appears from the above data that additional molecular events other than *RET* or *RAS* mutations are needed for the aggressive behavior seen in high grade MTC. In that regard, we herein reported *TP53* mutation as a novel prognostic molecular alteration in MTCs. *TP53* mutations was uncommon in MTCs, being reported in 9% (8/88 cases) in the literature [33] and 4.2% in our cohort. It may co-exist with *RET* or *RAS* driver mutations as shown in our cohort and in a previous publication [33]. The current study was the first to show that *TP53* mutation was associated with decreased OS, DSS, and LRRFS in MTC.

This international multicentric MTC consortium allowed us to provide detailed clinical, pathologic, and molecular characteristics of MTC. We have previously established and validated a prognostically relevant grading scheme [2]. We herein presented evidence that *RET* somatic mutations and *TP53* also carried prognostic values in MTC, although (possibly) not independent of grade and AJCC prognostic group. The next step is to establish a nomogram incorporating these prognostic factors, in order to better risk stratify patients with MTCs.

Table 1. Clinicopathologic characteristics according to *RET* and *RAS* mutation status.

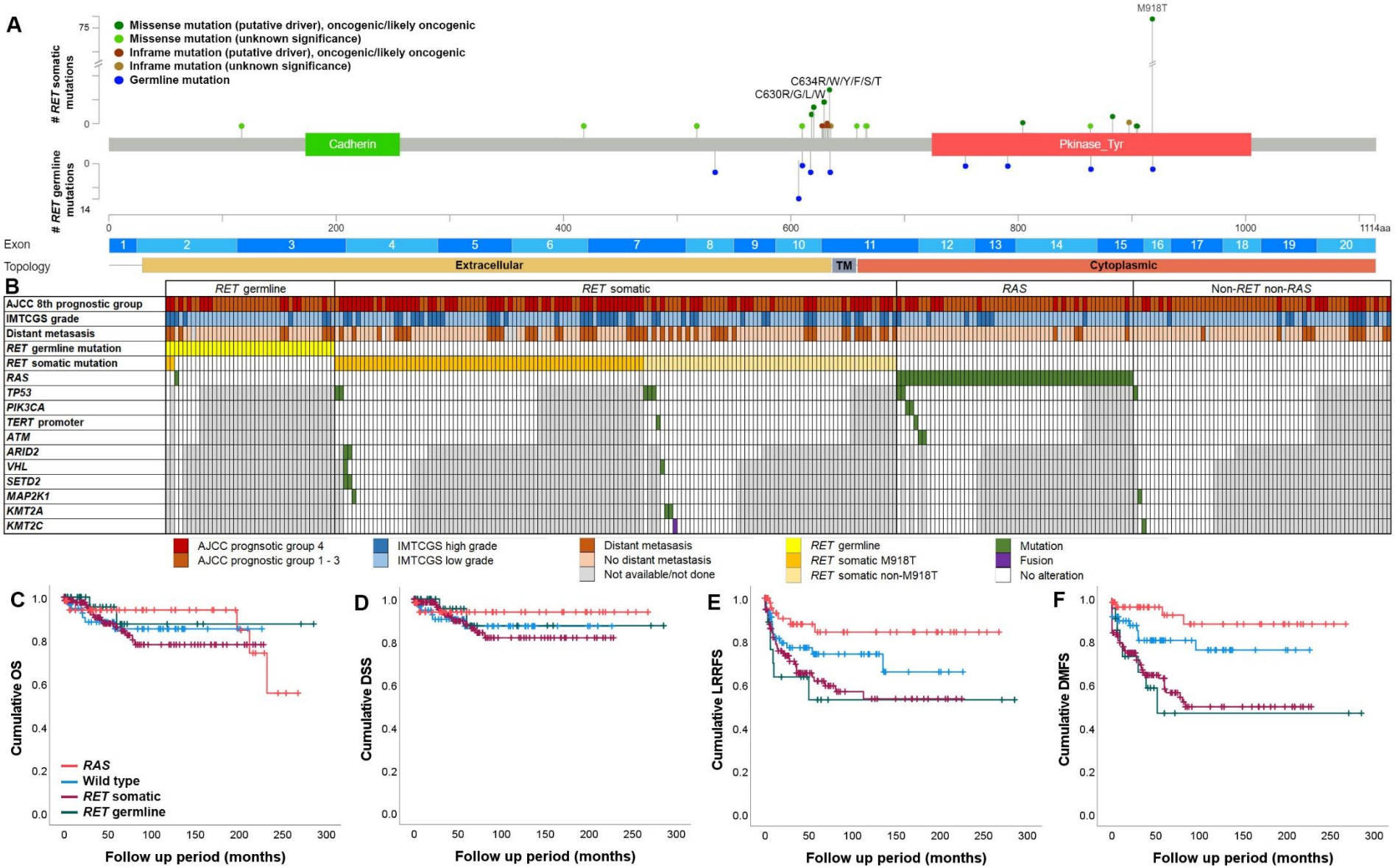
	All cases (N=290)	*RET* germline mutations (n=40)	*RET* somatic mutations (n=133)	*RAS* somatic mutations (n=56)	*RET/RAS* wild type (WT, n=61)	P values (*RET* somatic vs. WT)	P values (*RET* germline vs. WT)	P values (*RAS* vs. WT)	P values (*RET* somatic vs. RET germline)
Male: female ratio	138:152 (1:1.1)	18:22 (1:1.2)	77:56 (1:0.7)	18:38 (1:2.1)	35:36 (1:1.4)	0.031	NS	NS	NS
Age (years)	57 (7-88)	42 (7-66)	59 (22-88)	57 (7-79)	58 (25-84)	NS	<0.001	NS	<0.001
Tumor size (cm)	2.0 (0.1-11.0)	1.2 (0.1-6.0)	2.1 (0.4-8.0)	2.0 (0.3-11.0)	1.9 (0.3-6.0)	0.036	NS	NS	0.006
AJCC prognostic group						<0.001	NS	NS	0.006
1	92 (31.7%)	13 (32.5%)	29 (21.3%)	24 (42.9%)	26 (42.6%)				
2	43 (14.8%)	4 (10.0%)	13 (9.6%)	12 (21.4%)	14 (23.0%)				
3	41 (14.1%)	11 (27.5%)	17 (12.5%)	7 (12.5%)	6 (9.8%)				
4	114 (39.3%)	12 (30.0%)	77 (56.6%)	13 (23.2%)	15 (24.6%)				
Vascular invasion	122 (42.1%)	12 (30.0%)	76 (55.9%)	17 (30.4%)	20 (32.8%)	0.005	NS	NS	0.007
Extrathyroidal extension	83 (28.8%)	9 (22.5%)	47 (34.6%)	14 (25.0%)	15 (25.4%)	NS	NS	NS	NS
Positive margin	54 (18.6%)	5 (12.5%)	32 (23.5%)	11 (19.6%)	8 (13.1%)	NS	NS	NS	NS
First post-op CEA	5 (0-38335)	2 (0-600)	7 (0-26300)	5 (1-539)	3 (0-38335)	NS	NS	NS	NS
First post-op calcitonin	13 (1-970000)	48 (1-7979)	24 (1-970000)	5 (1-68791)	4 (1-16000)	NS	NS	NS	NS
Calcitonin doubling time									
Never doubled	105/163 (64.4%)	23/32 (71.9%)	40/75 (53.3%)	19/27 (70.4%)	23/31 (74.2%)	NS	NS	NS	NS
Months if doubled	17 (3-139)	13 (3-139)	15 (3-84)	27 (8-35)	15 (3-99)	NS	NS	NS	NS
IMTCGS grade						0.027	NS	NS	NS
Low grade	218 (75.2%)	33 (82.5%)	88 (64.7%)	47 (83.9%)	50 (82.0%)				
High grade	72 (24.8%)	7 (17.5%)	48 (35.3%)	9 (16.1%)	11 (18.0%)				
Necrosis	43 (14.8%)	1 (2.5%)	30 (22.1%)	4 (7.1%)	8 (13.1%)	NS	NS	NS	0.002
Mitotic index (/2mm^2)	1 (0-29)	0 (0-7)	1 (0-18)	1 (0-9)	1 (0-7)	0.028	NS	NS	0.049
KI67 (%)	2 (0-58)	1 (0-15)	2 (0-30)	2 (0-58)	2 (0-30)	NS	NS	NS	0.016
DM at presentation	25 (8.7%)	1 (2.5%)	19 (14.4%)	1 (1.8%)	4 (6.7%)	NS	NS	NS	0.047
Radiation therapy	21 (8.2%)	4 (10.3%)	14 (12.2%)	1 (2.1%)	2 (3.7%)	NS	NS	NS	NS
TKI	25 (8.7%)	2 (5.0%)	20 (15.2%)	1 (1.8%)	2 (3.3%)	0.015	NS	NS	NS

* The three cases with both *RET* germline mutations and somatic *RET* or *RAS* mutations are classified as *RET* germline mutations in this table.
Values are expressed as n (column %) for categorical variables, or median (range) for continuous variables.
NS: not significant. DM: distant metastasis,

Table 2. Impact of *RET* and *RAS* mutation on prognosis in medullary thyroid carcinoma. Values in the table are p values. Bold values: significant p values. OS: overall survival, DSS: disease specific survival, DMFS: distant metastasis free survival, LRRFS: locoregional recurrence free survival, WT: wild type.

	OS	**DSS**	**DMFS**	**LRRFS**
RET somatic vs. *RET* germline	0.387	0.556	0.745	0.630
RET somatic vs. *RET/RAS* WT	0.748	0.854	**0.010**	0.118
RET germline vs. *RET/RAS* WT	0.422	0.557	**0.045**	0.156
RAS mutated vs. *RET/RAS* WT	0.406	0.407	0.081	0.193
RET M918T somatic vs. other *RET* somatic	0.202	0.328	0.375	0.531

Figure 1. *RET* germline and somatic mutations in medullary thyroid carcinoma (MTC) and their impact on clinical outcome. (A) lollipop plot. Cadherin: Cadherin domain. Pkinase_Tyr: protein tyrosine kinase. TM: Transmembrane. (B) Oncoprint showing the clinicopathologic features and mutation profile of MTCs. (C-F) Kaplan Meier curves for overall survival (OS, C), disease specific survival (DSS, D), locoregional recurrence free survival (LRRFS, E), and distant metastasis free survival (DMFS, F). *RET* mutations, somatic or germline, are associated with decreased LRRFS and DMFS. OS and DSS do not differ according to *RET* or *RAS* mutation status.



Supplementary table 1. Next generation sequencing molecular platforms and genes tested with each platform.

Platforms	**Gene tested**
A custom-designed multi-gene panel	26 oncogenes or tumor suppressor genes as described previously [14], including *BRAF, c-KIT, CTNNB1, DICER1, DPYD, EGFR, EIF1AX, GNA11, GNAQ, GNAS, H3F3A, HRAS, IDH1, IDH2, KRAS, MED12, MET, NRAS, PDGFRa, PIK3CA, PTEN, RET, RNF43, SMAD4, TERT promoter, TP53, TSHR*, and *VHL*.
MSK-IMPACT	505 cancer-related genes as described previously [13,15].
Ion AmpliSeq Cancer Hotspot Panel v2 (CHP2)	Commercially available platform of 50 oncogenes and tumor suppressor genes and four fusions, including *ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, EZH2, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, NTRK1, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, ROS1, SMAD4, SMARCB1, SMO, SRC, STK11, TP53,* and *VHL*.
OncoPanel	298 to ~500 genes as described previously [12,17].
Paradigm Cancer Genetics Platform	A commercially available platform of 230 genes as described previously [16].
CARIS NGS platform	A commercially available whole exome sequencing platform.

Supplementary Table 2. *RET* germline and somatic mutations in medullary thyroid carcinoma. ATA: American Thyroid Association.

***RET* mutations**	**n**	**ATA risk (for germline mutation) or OncoKB annotation (for somatic mutation)**
Germline mutations (40/246, 16.3%)		
M918T	2	ATA highest
V804L	2	ATA modest
V804M	1	ATA modest
Y791F	1	ATA modest
C634R	8	ATA high
C634F	2	ATA high
C634Y	1	ATA high
C618R	1	ATA modest
C618Y	1	ATA modest
G611Y	1	ATA modest
C611S	1	ATA modest
C609Y	14	ATA modest
G533C	3	ATA modest
Exon 11	1	N/A
Somatic mutations (135/290, 46.6%)		
M918T	73	Oncogenic
M918T, G601R	1	Oncogenic, unknown
M918T, P516L	1	Oncogenic, unknown
S904A, D898_E901del	1	Likely oncogenic, unknown
S904P, D898_E901del	1	Likely oncogenic, unknown
D898_E901del	1	Unknown
A883F	6	Oncogenic
G861Q	1	Unknown
V804M	3	Oncogenic
K666E	1	Unknown
K666N	1	Unknown
C634R	8	Oncogenic
C634W	3	Likely oncogenic
C634Y	1	Likely oncogenic
C634F	1	Likely oncogenic
C634S	1	Likely oncogenic
C634T	1	Likely oncogenic
C634_I638del	1	Unknown
E632_C634del	1	Likely oncogenic
E632_L633del, H658Y	1	Likely oncogenic, unknown
D631_C634del	1	Likely oncogenic
D631_L633delinsE	1	Likely oncogenic
C630R	5	Oncogenic
C630G	3	Likely oncogenic

C630L	1	Likely oncogenic
C630_D631del	1	Likely oncogenic
C630W	1	Likely oncogenic
L629_L633del	1	Likely oncogenic
C620R	3	Oncogenic
C620S	3	Likely oncogenic
C620Y	1	Likely oncogenic
C618A	1	Likely oncogenic
C618G	1	Likely oncogenic
C618R	1	Oncogenic
C611S	1	Likely oncogenic
R417P	1	Unknown
R112C	1	Unknown

Supplementary table 3. Clinicopathologic features of medullary thyroid carcinoma with *RET* M918T somatic mutations and those with other *RET* somatic mutations. NS: not significant

	RET M918T somatic mutation (n=75)	*RET* non-M918T somatic mutation (n=60)	P values
Male: female ratio	44:31 (1.4:1)	34:26 (1.3:1)	NS
Age (years)	52 (22-84)	63 (27-88)	<0.001
Tumor size (cm)	2.1 (0.4-8.0)	2.0 (0.5-9.0)	NS
AJCC prognostic group			<0.001
1	10 (13.3%)	19 (31.7%)	
2	6 (8.0%)	7 (11.7%)	
3	7 (9.3%)	10 (16.7%)	
4	52 (69.3%)	24 (40.0%)	
Vascular invasion	49 (65.3%)	26 (43.3%)	0.015
Extrathyroidal extension	35 (46.7%)	12 (20.0%)	0.002
Positive margin	23 (30.7%)	9 (15.0%)	0.042
First post-op CEA	15 (0-26300)	4 (1-1152)	NS
First post-op calcitonin	83 (1-970000)	6 (1-4481)	NS
Calcitonin doubling time			
Never doubled	20/41 (48.8%)	20/33 (60.6%)	NS
Months if doubled	18 (3-84)	10 (4-57)	NS
Grade			NS
Low grade	46 (61.3%)	42 (70.0%)	
High grade	29 (38.7%)	18 (30.0%)	
Necrosis	16 (21.3%)	14 (23.3%)	NS
Mitotic index (/2mm^2)	1 (0-18)	1 (0-15)	NS
KI67 (%)	3 (0-30)	2 (0-25)	NS
Distant metastasis at presentation	8 (10.8%)	11 (18.3%)	NS
Radiation therapy	6 (9%)	9 (17.6%)	NS
Kinase inhibitor therapy	15 (20.3%)	6 (10%)	NS

Supplementary table 4. Multivariate survival analysis for distant metastasis free survival using Cox proportional hazards model. Bold p values: significant p values.

	P values	Hazard ratio (95% confidence interval)
RET or *RAS* mutations		
RET and *RAS* wild type	Reference	
RET germline mutations	0.154	1.945 (0.779-4.856)
RET somatic mutations	0.217	1.525 (0.780-2.979)
RAS somatic mutation	0.147	0.428 (0.136-1.349)
IMTCGS grade	**0.006**	2.031 (1.230-3.353)
AJCC stage group 4	**<0.001**	1.478 (1.221-1.789)

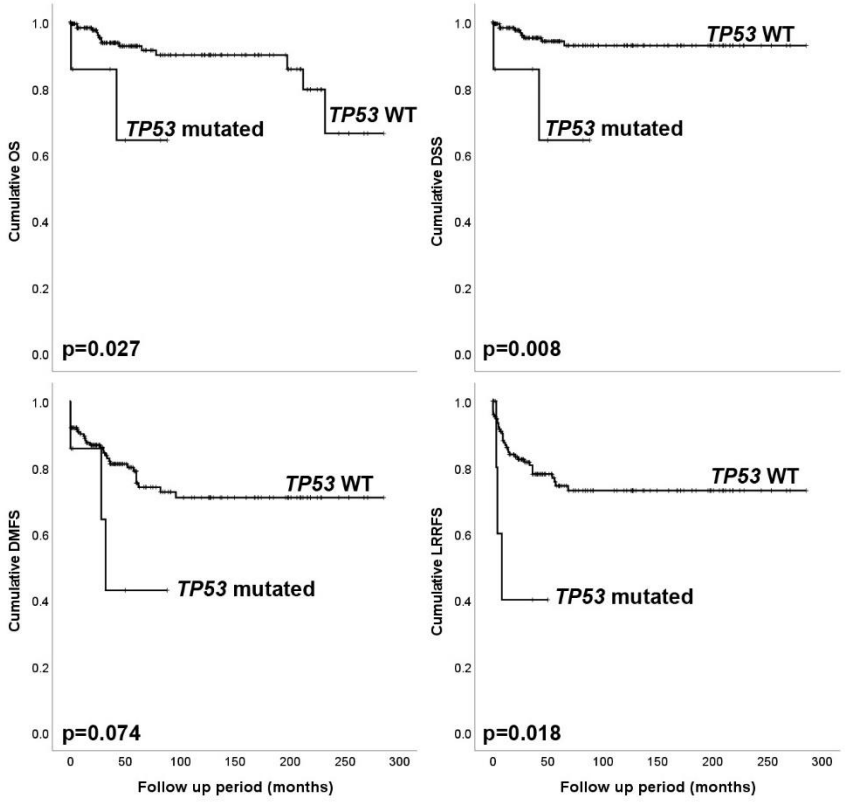
Supplementary Table 5: Other molecular alterations in medullary thyroid carcinoma.

RET or RAS somatic mutations	**Other molecular alterations**
RET M918T	*TP53* G360W
RET M918T	*TP53* V197E
RET M918T	*MGA* W2174*, *DIS3* R689Q
RET M918T	*FOXA1* P71Dfs*130, *FANCA* S858R
RET M918T	*ATM* R3008H, *SETD2* Y1666C, *PIK3CB* R321Q
RET M918T	*ARID2* Q1062*, *SETD2* R1625C
RET M918T*, RET* G601R	*MAPK1* E322K
RET S904P*, RET* D898_E901del	*RPS6KA4* S236L
RET D898_E901del	*KMT2C::HILPDA* fusion
RET A883F	*VHL* W117dup
RET V804M	*TP53* R156C
RET C634R	*KMT2A* M1585Ifs*7
RET C634R	*ARID2* F233Lfs*59, *MUTYH* R95W
RET C634R	*RB1* H339_D340fs
RET C634F	*TP63* R379C, *EP300* D1399N, *CHEK2* R346C
RET C634_I638del	*TGFBR1* E125Dfs*19
RET L629_L633del	*ARID1A* S2096*
RET C620Y	*APC* R854*
RET C618R	*TP53* V172D
RET C618G	*KMT2A* K461Nfs*106
NRAS Q61K	*TERT* c.-124 C>T
KRAS G12V	*PIK3CA* R108H
KRAS D54N	*TERT* c.-124 C>T
HRAS Q61R	*TP53* Q317*
HRAS Q61R	*SMAD4* L200Pfs*6
HRAS Q61R	*PIK3CA* D1017N
HRAS Q61K	*TP53* R248W, *KMT2C* R5432W, *SMAD2* R321Q
HRAS G12V	*EGFR* T790M, *VHL* R161Q, *RAD50* D69N, *SPEN* M612Cfs*2, *MAX* R60Q
-	*MAP2K1* K57N
-	*MAP2K1* E102_I103del
-	*KMT2C* A1685S
-	*GNAS* R201C

-	*ATM* R337C
-	*TP53* T256I
-	*ALK* R395H, *MST1* G673S, *MST1* R651*, *FAT1* R1205*, *LZTR1* R340*
-	*IDH2* R140Q, *FOXF1* G162S

-: negative for *RET* and *RAS* mutations.

Supplementary Figure 1. *TP53* mutation is associated with decreased survival in medullary thyroid carcinoma. Kaplan Meier curves for overall survival (OS), disease specific survival (DSS), distant metastasis free survival (DMFS), and locoregional recurrence free survival (LRRFS). WT: wild type.



References

1. Barletta JA, Nosé V, Sadow PM. Genomics and Epigenomics of Medullary Thyroid Carcinoma: From Sporadic Disease to Familial Manifestations. Endocrine pathology 2021;32(1):35-43, doi:10.1007/s12022-021-09664-3
2. Xu B, Fuchs TL, Ahmadi S, et al. International Medullary Thyroid Carcinoma Grading System: A Validated Grading System for Medullary Thyroid Carcinoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 2022;40(1):96-104, doi:10.1200/JCO.21.01329
3. Mohammadi M, Hedayati M. A Brief Review on The Molecular Basis of Medullary Thyroid Carcinoma. Cell journal 2017;18(4):485-492
4. 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 : official journal of the American Thyroid Association 2005;15(6):531-44, doi:10.1089/thy.2005.15.531
5. Elisei R, Tacito A, Ramone T, et al. Twenty-Five Years Experience on RET Genetic Screening on Hereditary MTC: An Update on The Prevalence of Germline RET Mutations. Genes (Basel) 2019;10(9), doi:10.3390/genes10090698
6. Fussey JM, Vaidya B, Kim D, et al. The role of molecular genetics in the clinical management of sporadic medullary thyroid carcinoma: A systematic review. Clin Endocrinol (Oxf) 2019, doi:10.1111/cen.14060
7. Ciampi R, Romei C, Ramone T, et al. Genetic Landscape of Somatic Mutations in a Large Cohort of Sporadic Medullary Thyroid Carcinomas Studied by Next-Generation Targeted Sequencing. iScience 2019;20(324-336, doi:10.1016/j.isci.2019.09.030
8. Romei C, Casella F, Tacito A, et al. New insights in the molecular signature of advanced medullary thyroid cancer: evidence of a bad outcome of cases with double RET mutations. J Med Genet 2016;53(11):729-734, doi:10.1136/jmedgenet-2016-103833
9. Ciampi R, Mian C, Fugazzola L, et al. Evidence of a low prevalence of RAS mutations in a large medullary thyroid cancer series. Thyroid : official journal of the American Thyroid Association 2013;23(1):50-7, doi:10.1089/thy.2012.0207
10. Boichard A, Croux L, Al Ghuzlan A, et al. Somatic RAS mutations occur in a large proportion of sporadic RET-negative medullary thyroid carcinomas and extend to a previously unidentified exon. The Journal of clinical endocrinology and metabolism 2012;97(10):E2031-5, doi:10.1210/jc.2012-2092
11. Wirth LJ, Sherman E, Robinson B, et al. Efficacy of Selpercatinib in RET-Altered Thyroid Cancers. The New England journal of medicine 2020;383(9):825-835, doi:10.1056/NEJMoa2005651
12. Garcia EP, Minkovsky A, Jia Y, et al. Validation of OncoPanel: A Targeted Next-Generation Sequencing Assay for the Detection of Somatic Variants in Cancer. Archives of pathology & laboratory medicine 2017;141(6):751-758, doi:10.5858/arpa.2016-0527-OA
13. Cheng DT, Mitchell TN, Zehir A, et al. Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT): A Hybridization Capture-Based Next-Generation Sequencing Clinical Assay for Solid Tumor Molecular Oncology. The Journal of molecular diagnostics : JMD 2015;17(3):251-64, doi:10.1016/j.jmoldx.2014.12.006
14. de Biase D, Acquaviva G, Visani M, et al. Molecular Diagnostic of Solid Tumor Using a Next Generation Sequencing Custom-Designed Multi-Gene Panel. Diagnostics (Basel) 2020;10(4), doi:10.3390/diagnostics10040250
15. Zehir A, Benayed R, Shah RH, et al. Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients. Nat Med 2017;23(6):703-713, doi:10.1038/nm.4333
16. Weiss GJ, Hoff BR, Whitehead RP, et al. Evaluation and comparison of two commercially available targeted next-generation sequencing platforms to assist oncology decision making. OncoTargets and therapy 2015;8(959-67, doi:10.2147/ott.S81995
17. Lim-Fat MJ, Youssef GC, Touat M, et al. Clinical utility of targeted next-generation sequencing assay in IDH-wildtype glioblastoma for therapy decision-making. Neuro Oncol 2022;24(7):1140-1149, doi:10.1093/neuonc/noab282
18. Wells SA, Jr., Asa SL, Dralle H, et al. Revised American Thyroid Association guidelines for the management of medullary thyroid carcinoma. Thyroid : official journal of the American Thyroid Association 2015;25(6):567-610, doi:10.1089/thy.2014.0335
19. 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(19):1575-9
20. Machens A, Niccoli-Sire P, Hoegel J, et al. Early malignant progression of hereditary medullary thyroid cancer. The New England journal of medicine 2003;349(16):1517-25, doi:10.1056/NEJMoa012915
21. Maciel RMB, Camacho CP, Assumpção LVM, et al. Genotype and phenotype landscape of MEN2 in 554 medullary thyroid cancer patients: the BrasMEN study. Endocr Connect 2019;8(3):289-298, doi:10.1530/ec-18-0506

22. Mian C, Pennelli G, Barollo S, et al. Combined RET and Ki-67 assessment in sporadic medullary thyroid carcinoma: a useful tool for patient risk stratification. European journal of endocrinology / European Federation of Endocrine Societies 2011;164(6):971-6, doi:10.1530/EJE-11-0079
23. 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. The Journal of clinical endocrinology and metabolism 2008;93(3):682-7, doi:10.1210/jc.2007-1714
24. Vuong HG, Odate T, Ngo HTT, et al. Clinical significance of RET and RAS mutations in sporadic medullary thyroid carcinoma: a meta-analysis. Endocrine-related cancer 2018;25(6):633-641, doi:10.1530/erc-18-0056
25. Xing M, Alzahrani AS, Carson KA, et al. Association between BRAF V600E mutation and mortality in patients with papillary thyroid cancer. Jama 2013;309(14):1493-501, doi:10.1001/jama.2013.3190
26. Najdawi F, Ahmadi S, Capelletti M, et al. Evaluation of grade in a genotyped cohort of sporadic medullary thyroid carcinomas. Histopathology 2021;79(3):427-436, doi:10.1111/his.14370
27. Schilling T, Burck J, Sinn HP, et al. Prognostic value of codon 918 (ATG-->ACG) RET proto-oncogene mutations in sporadic medullary thyroid carcinoma. International journal of cancer Journal international du cancer 2001;95(1):62-6, doi:10.1002/1097-0215(20010120)95:1<62::aid-ijc1011>3.0.co;2-1
28. Romei C, Ugolini C, Cosci B, et al. Low prevalence of the somatic M918T RET mutation in micro-medullary thyroid cancer. Thyroid : official journal of the American Thyroid Association 2012;22(5):476-81, doi:10.1089/thy.2011.0358
29. 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(11):1777-83, doi:10.1038/sj.bjc.6605056
30. Agrawal N, Jiao Y, Sausen M, et al. Exomic sequencing of medullary thyroid cancer reveals dominant and mutually exclusive oncogenic mutations in RET and RAS. The Journal of clinical endocrinology and metabolism 2013;98(2):E364-9, doi:10.1210/jc.2012-2703
31. Simbolo M, Mian C, Barollo S, et al. High-throughput mutation profiling improves diagnostic stratification of sporadic medullary thyroid carcinomas. Virchows Archiv : an international journal of pathology 2014;465(1):73-8, doi:10.1007/s00428-014-1589-3
32. Heilmann AM, Subbiah V, Wang K, et al. Comprehensive Genomic Profiling of Clinically Advanced Medullary Thyroid Carcinoma. Oncology 2016;90(6):339-46, doi:10.1159/000445978
33. Minna E, Romeo P, Dugo M, et al. Medullary Thyroid Carcinoma Mutational Spectrum Update and Signaling-Type Inference by Transcriptional Profiles: Literature Meta-Analysis and Study of Tumor Samples. Cancers 2022;14(8), doi:10.3390/cancers14081951