Genomic and Transcriptomic Landscape of *RET* Wild-Type Medullary Thyroid Cancer and Potential Use of Mitogen-Activated Protein Kinase-Targeted Therapy

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BACKGROUND: About 75% of medullary thyroid cancers (MTCs) are sporadic with 45% to 70% being driven by a *RET* mutation. Selpercatinib is an approved treatment for RET-mutated (mut*RET*) MTC; however, treatments are needed for wild-type RET MTC (wt*RET*). Genomic alterations and transcriptomic signatures of wt*RET* MTC may reveal new therapeutic insights.

STUDY DESIGN: We did a retrospective analysis of MTC samples submitted for DNA/RNA sequencing and programmed cell death ligand 1 expression using immunohistochemistry at a Clinical Laboratory Improvement Amendments/College of American Pathologists-certified laboratory. Tumor microenvironment immune cell fractions were estimated using RNA deconvolution (quanTIseq). Transcriptomic signatures of inflammation and MAP kinase pathway activation scores were calculated. Mann-Whitney U, chi-square, and Fisher's exact tests were applied (p values adjusted for multiple comparisons).

RESULTS: The 160-patient cohort included 108 mut*RET* and 52 wt*RET* MTC samples. wt*RET* tumors frequently harbored mitogen-activated protein kinase (MAPK) pathway mutations, including *HRAS* (42.31%), *KRAS* (15.7%), *NF1* (6.7%), and *BRAF* (2%), whereas only 1 MAPK pathway mutation (*NF1*) was identified among mut*RET* MTC. Recurrent mutations seen in wt*RET* MTC included *MGA*, *VHL, APC*, *STK11*, and *NFE2L2*. Increased transcriptional activation of the MAPK pathway was observed in patients with wt*RET* harboring mutations in MAPK genes. Although the frequency of programmed cell death ligand 1 expression was similar in wt*RET* and mut*RET* (10.2% vs 7%, p = 0.531), wt*RET* tumors were more often tumor mutational burden high (7.7% vs 0%, p = 0.011), and wt*RET* MTC exhibited higher expression of immune checkpoint genes.

CONCLUSIONS: We identified molecular alterations and immune-related features that distinguish wt*RET* from mut*RET* MTC. Although *RET* mutation drives MTC in the absence of other alterations, we showed that wt*RET* MTC frequently harbors MAPK pathway mutations. These findings may indicate a potential basis for MAPK-targeted therapy, possibly in combination with immuno-oncology agents for selected patients with wt*RET* MTC. (J Am Coll Surg 2024;239:50–60.

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Abbreviations and Acronyms

dMMR/MSI-H	=	deficient mismatch repair and microsatellite instability-high
FFPE	=	formalin fixed paraffin embedded
GSEA	=	gene set enrichment analysis
HR	=	hazard ratio
MAPK	=	mitogen-activated protein kinase
MPAS	=	MAPK pathway activation score
MTC	=	medullary thyroid cancer
mut*RET*	=	RET mutated
NGS	=	next-generation sequencing
OS	=	overall survival
PD-L1	=	programmed cell death ligand 1
TKI	=	tyrosine kinase inhibitor
TMB	=	tumor mutational burden
TME	=	tumor microenvironment
wt*RET*	=	wild-type RET

Medullary thyroid carcinoma (MTC) is a rare neuroendocrine tumor arising from calcitonin-secreting parafollicular cells (C cells) with a low incidence rate (0.14 to 0.21/100,000) and representing approximately 2% to 4% of all thyroid cancer cases.[1] Approximately 25% of MTC are related to a hereditary syndrome and 75% occur on a sporadic basis. Hereditary syndromes predisposing a patient to MTC include multiple endocrine neoplasia type 2A, multiple endocrine neoplasia type 2B (more aggressive MTC at an earlier age, pheochromocytomas and neuromas), and familial (nonmultiple endocrine neoplasia) MTC.[2] Among sporadic MTC, 45% to 70% of cases have been attributed to an activating point mutation in the receptor tyrosine kinase gene *RET*, an oncogenic driver implicated in other cancers as well.[3,4] Although a majority of *RET* mutations in sporadic MTC affect codon 918 (M918T), followed by codon 634, other mutations have been identified as well.[2,5-12]

In 2011 and 2012, the FDA approved the use of 2 multikinase inhibitors, vandetinib and cabozantinib, for the treatment of advanced or metastatic MTC.[13] Treatment with either agent improved progression-free survival.[14,15] Then, in 2021, selpercatenib, a highly selective *RET* tyrosine kinase inhibitor (TKI), was approved for the treatment of *RET*-mutant MTCs and *RET* fusion differentiated thyroid cancer. The response rate in treatment-naive, unresectable or recurrent RET-mutated MTC (mut*RET*) to selpercatenib was reported to be 73%, and for patients previously treated with vandetinib or cabozantinib, the response rate was 79%.[4] Other therapeutic agents, however, are needed to treat 30% to 55% of patients with RET wild-type (wt*RET*) MTC.

Although *RAS* mutations (*HRAS*, *KRAS*, *NRAS*) are reported to underlie about 70% of these MTC cases,[16-19] we analyzed a large cohort of patient samples with MTC to identify molecular features of wt*RET* MTC that may provide new therapeutic options for these patients. Herein, we show that subpopulations of patients with wt*RET* MTC have activation of the mitogen-activated protein kinase (MAPK) pathway or an immune profile that suggests potential benefit from MAPK-targeted and immunotherapy treatment.

METHODS

Study cohort

Formalin-fixed paraffin-embedded (FFPE) samples from patients with MTC (160) were submitted to a commercial Clinical Laboratory Improvement Amendments/College of American Pathologists-certified laboratory for molecular profiling (Caris Life Sciences, Phoenix, AZ). The study follows guidelines provided by the Declaration of Helsinki, Belmont Report, and US Common Rule. In accordance with compliance policy 45 CFR 46.101(b), this study was conducted using retrospective, de-identified clinical data, patient consent was not required, and the study was considered IRB exempt.

DNA next-generation sequencing

In preparation of the samples for molecular testing, tumor enrichment was performed by harvesting targeted tissues using manual microdissection techniques. Genomic DNA was extracted from FFPE tissue samples and subjected to next-generation sequencing (NGS) using the NextSeq or NovaSeq 6000 Platforms (Illumina, Inc, San Diego, CA). A custom SureSelect XT assay (Agilent Technologies, Santa Clara, CA) was used to enrich exonic regions of 592 whole-gene targets. For tumor samples sequenced on NovaSeq 6000 platform (n = 133), more than 700 clinically relevant genes were assessed. All variants were detected with >99% confidence based on allele frequency and amplicon coverage, with an average sequencing depth of coverage of >500 and an analytic sensitivity threshold established of 5% for variant calling. Certified molecular geneticists examined the identified genomic variants and categorized them in alignment with the standards set by the American College of Medical Genetics and Genomics. Calculation of mutation frequencies in individual genes included "pathogenic" and "likely pathogenic" variants, whereas those labeled as "benign," "likely benign," and "variants of unknown significance" were excluded.

Tumor mutational burden

Tumor mutational burden (TMB) was measured by counting all nonsynonymous missense, nonsense, in-frame

insertion/deletion, and frameshift mutations found per tumor that had not been previously described as germline alterations in dbSNP151, Genome Aggregation Database, or benign variants identified by Caris's geneticists. High TMB (TMB-H) was defined by a cutoff value of 10 mut/MB or more based on the KEYNOTE-158 pembrolizumab trial, in which it was shown that patients with 10 mut/MB or more had increased response rates compared with those with less than 10 mut/MB.[20]

Whole transcriptomic sequencing

FFPE specimens underwent pathology review to measure percent tumor content and tumor size; a minimum of 10% of tumor content in the area for microdissection was required to enable enrichment and extraction of tumor-specific RNA. Qiagen RNA FFPE tissue extraction kit was used for extraction, and the RNA quality and quantity were determined using the Agilent TapeStation. Biotinylated RNA baits were hybridized to the synthesized and purified cDNA targets, and the bait-target complexes were amplified in a postcapture polymerase chain reaction. The Illumina NovaSeq 6500 was used to sequence the whole transcriptome from patients to an average of 60M reads. Raw data were demultiplexed by an Illumina Dragen BioIT accelerator, trimmed, counted, polymerase chain reaction duplicates removed and aligned to human reference genome hg19 by STAR aligner. For transcription counting, transcripts per million values were generated using the Salmon expression pipeline.

Gene expression profiling and signatures

Gene set enrichment analysis (GSEA)[21] was carried out using the WTS data and the Hallmark gene set collection from the Human Molecular Signatures Database.[22] A MAPK pathway activation score (MPAS), which serves as a transcriptomic measure of the activation state of the MAPK pathway, was calculated as the average z-score of expression values (in transcripts per million units) for a set of 10 genes (*SPRY2, SPRY4, ETV4, ETV5, DUSP4, DUSP6, CCND1, PHLDA1, EPHA2,* and *EPHA4*), as previously described.[23] Immune cell fraction was calculated using the quanTIseq pipeline, which used deconvolution of bulk transcriptomic data.[24]

Immunohistochemistry

Immunohistochemistry was conducted on complete sections of FFPE tissues mounted on glass slides. The slides underwent staining using automated staining methods as directed by the manufacturer. These procedures were meticulously optimized and confirmed to meet the standards outlined by Clinical Laboratory Improvement Amendments/College of American Pathologists and International Organization for Standardization. Programmed cell death ligand 1 (PD-L1) expression was determined using primary antibody SP142 (Spring Biosciences, Pleasanton, CA), with a positive threshold of ≥2+ stain intensity and ≥5% percentage of cells stained.

Deficient mismatch repair and microsatellite instability-high

Deficient mismatch repair and microsatellite instability-high (dMMR/MSI-H) was determined by a combination of immunohistochemistry using antibodies for MLH1 (M1 antibody), MSH2 (G2191129 antibody), MSH6 (44 antibody), and PMS2 (EPR3947 antibody) from Ventana Medical Systems (Tucson, AZ), and NGS. The outcomes from these 3 platforms are mostly in agreement, as previously described.[25] In instances where conflicting results emerged, the order of priority for determining the MSI/MMR status of the tumor was immunohistochemistry, followed by NGS.

Real-world overall survival data

Real-world overall survival (OS) information was obtained from insurance claims data and calculated from either time of biopsy or start of therapy to last contact. Hazard ratio (HR) was calculated using the Cox proportional hazard models and p values were calculated using the log-rank test with statistical significance determined at a p value of <0.05.

Statistics

Comparative analysis of molecular alterations was assessed using Mann-Whitney U test, whereas categorical data were evaluated using chi-square or Fisher's exact test. The significance for the immune cell abundance in the tumor microenvironment (TME) across different cohorts was tested using a nonparametric Kruskal–Wallis test with post hoc pairwise comparison by the Mann-Whitney U test. The p values were adjusted for multiple hypotheses testing by the Benjamini–Hochberg method to reduce the risk of type 1 error. All statistical analyses were two-sided, with significance level set to 0.05.

RESULTS

Clinicodemographic characteristics

The study cohort of 160 patients comprised 63.1% men (101) and 36.9% women (59), with median age of 61 years (range 17 to 88) at the time of biopsy collection. In total, 67.5% (108) were mut*RET*, whereas 32.5% (52) were wt*RET* MTC samples (Table 1). Patient age was not

Table 1. Clinicodemographic Data of Patients with Medullary Thyroid Cancer

Characteristic	Overall	RET mutated	Wild-type RET	p Value
Total, n (%)	160	108 (67.5)	52 (32.5)	
Age, y, median (range)	61 (17–88)	60.5 (17–88)	62.5 (25–83)	0.068
Sex, n (%)				0.95
Male	101 (63.1)	68 (63)	33 (63.5)	
Female	59 (36.9)	40 (37)	19 (36.5)	
Specimen site, n (%)				
Primary				
Thyroid	80 (50)	49 (45.4)	31 (59.6)	
Metastatic				
Lymph node	50 (31.25)	37(34.3)	13 (25)	
Head neck	7 (4.4)	6 (5.5)	1 (1.9)	
Lung	6 (3.75)	4 (3.7)	2 (3.8)	
Other	17 (10.625)	12 (11.1	5 (9.6)	
Smoking status, n (%)				
Yes	5 (3.1)			
No smoking data	155 (96.9)			

associated with *RET* mutation status (median age 60.5 y for mut*RET* vs 62.5 y for wt*RET*; p = 0.068).

RET mutation in medullary thyroid cancer

Codon M918T, which is within the protein kinase domain of the *RET* gene was the most frequent mutation, observed in 59.2% (64) of the *RET*-mutated cases. Other *RET* mutations observed included C634R (5), C618R (4)—which affect the transmembrane domain of the *RET* gene—and A883F (4), found within the kinase domain (Fig. 1A). Prevalence of DNA repair gene mutations were similar between patients with mut*RET* or wt*RET* MTC (*ARID1A*: 0.9% vs 5.8%, p = 0.101; *BRCA1*: 0.0% vs 1.9%, p = 0.33; *ATM*: 1.9% vs 1.9%, p = 1.00). No mutations were observed in BRCA2 or ATR. Other recurrent mutations in wt*RET* MTC tumors included *MGA* (4, 13.8%), *VHL* (3, 5.8%), *APC* (3; 4%), *STK11* (2, 4%), *NFE2L2* (2, 4.3%). In addition to these observed mutations, we also observed patients with wt*RET* MTC harbored pathogenic *RET* fusion (*KIF5B-RET*; 1, 1.92%) and *NTRK2* fusion (*KCTD16-NTRK2*, *ETV6-NTRK2*; 2, 3.84%; Fig. 1B).

Mitogen-activated protein kinase–associated genes are relevant in medullary thyroid cancer

wt*RET* MTC tumors frequently harbored mutations in MAPK pathway genes, including *HRAS* (22, 42.31%), *KRAS* (8, 15.7%), *NF1* (3, 6.7%), and *BRAF* (1, 2%), whereas only 1 MAPK pathway mutation (*NF1*) was identified among mut*RET* MTC (variant allele frequency of *NF1* in this sample was 43%; however, matched normal tissue was not available to confirm a germline *NF1* mutation). The most common *HRAS* mutation was G61R (16 of 22), whereas others include G13R (5 of 22) and Q61K (1 of 22). *KRAS* mutations include Q61R (3 of 8), G12R (2 of 8), G12V (2 of 8), and Q61L (1 of 8). The only patient with a *BRAF* mutation harbored a V471F class II mutation. We divided the wt*RET* cohorts into 2 based on alterations in MAPK-associated genes (indel mutation/copy number amplification) and those without alterations (wt). We calculated the MAPK pathway activation using a quantitative scoring approach of our transcriptomic data. MAPK pathway activity score (MPAS) has been shown to be a prognostic biomarker of MAPK activity and predictive of response to TKIs. Our data revealed a statistically significant higher MPAS in the wt*RET* MTC cohort with MAPK alterations as compared with MAPK-WT (median MPAS: 0.49 vs −1.95, p = 0.015; Fig. 2A). However, we observed no significant difference in MPAS between the mut*RET* vs wt*RET* MTC cohort (median MPAS:0.38 vs −0.27, p = 0.081; Fig. 2A).

To investigate the relationship between *RET* mutational status and biological function through pathway enrichment analysis, we performed single-sample GSEA. Our analysis shows the distribution of normalized single-sample GSEA score of patients with different pathways (Fig. 2B). We then used GSEA to assess significantly enriched hallmark between the mut*RET* vs wt*RET*-MAPK-Altered vs wt*RET*-MAPK-WT MTC cohorts. Our result showed that significant enrichment of angiogenesis and *KRAS* downregulated the process in the wt*RET*-MAPK-WT

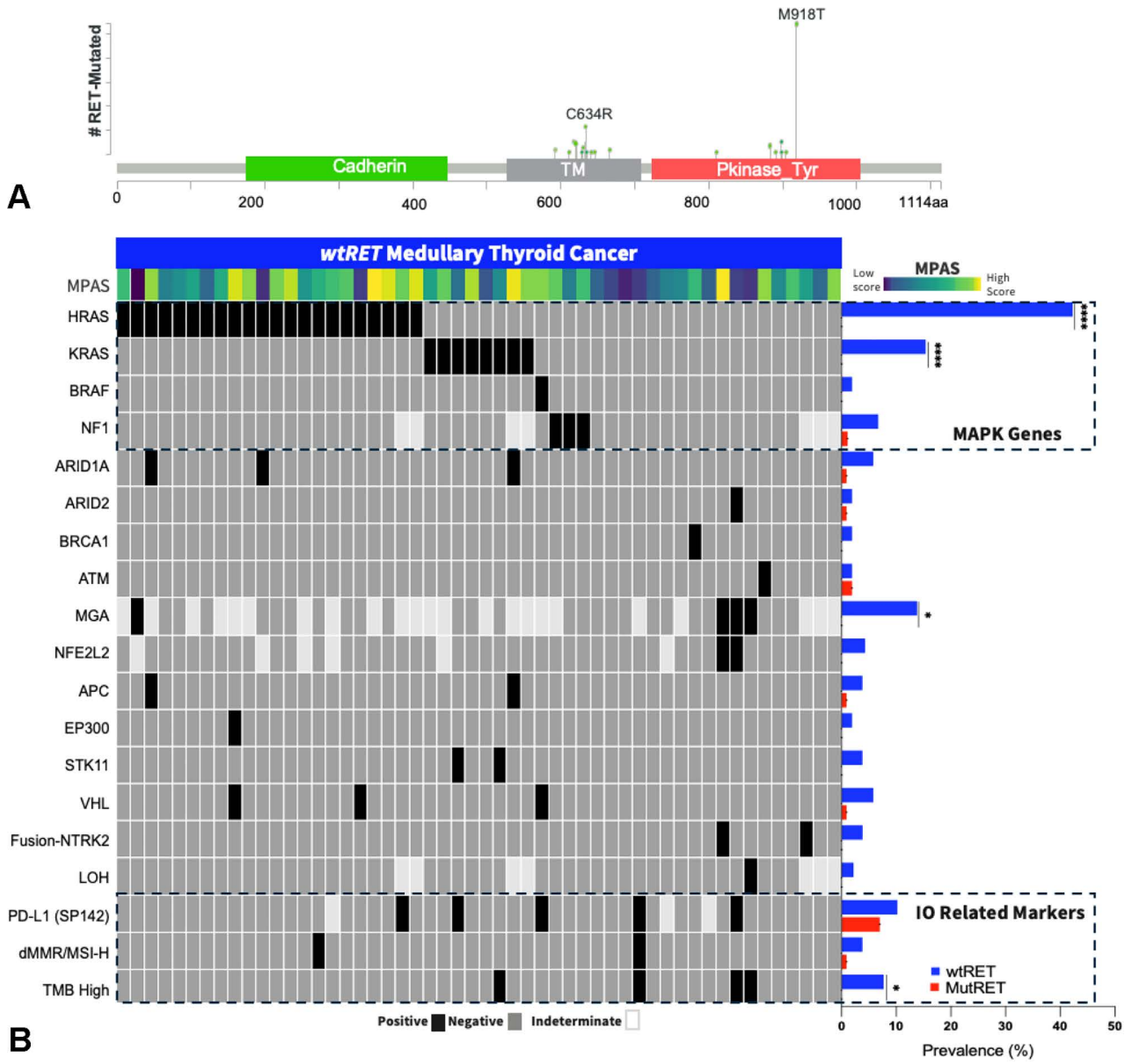

Figure 1. Mutational landscape of wt*RET* medullary thyroid cancer (A). Commonly mutated codons in *RET* gene (adapted from cBioPortal). (B) Oncoprint displays the pathogenic molecular alterations in wt*RET*, whereas the side bar graph displays a comparison of the prevalence between the wt*RET* and mut*RET* cohorts. mut*RET, RET* mutated; wt*RET*, wild-type *RET.*

compared with mut*RET* or wt*RET*-MAPK-Altered. We also observed E2F targets pathway is significantly enriched in the mut*RET* compared with wt*RET*-MAPK-WT (Fig. 2C).

Tumor microenvironment and predictors of response to immunotherapy in medullary thyroid cancer

We performed gene expression profiling to access the relationship between TME and *RET* mutation status. mut*RET* MTC showed significantly increased proportion of NK cells, CD4, and Tregs compared with wt*RET* (wt*RET*-MAPK-Altered and wt*RET*-MAPK-WT—NK cells median: 5.67 vs 5.33 vs 3.58, p = 0.0015; T cells CD4+ median: 2.51 vs 1.92 vs 0.00, p = 0.039; Tregs median: 3.12 vs 2.68, vs 2.35, p = 0.014). On the other hand, the proportion of macrophages M1 (median: 0.00 vs 0.72 vs 1.07, p = 0.018) and neutrophils (median: 0.00 vs 1.29 vs 2.71, p = 0.035) was significantly higher in the wt*RET* cohorts (Figs. 3A, 3B).

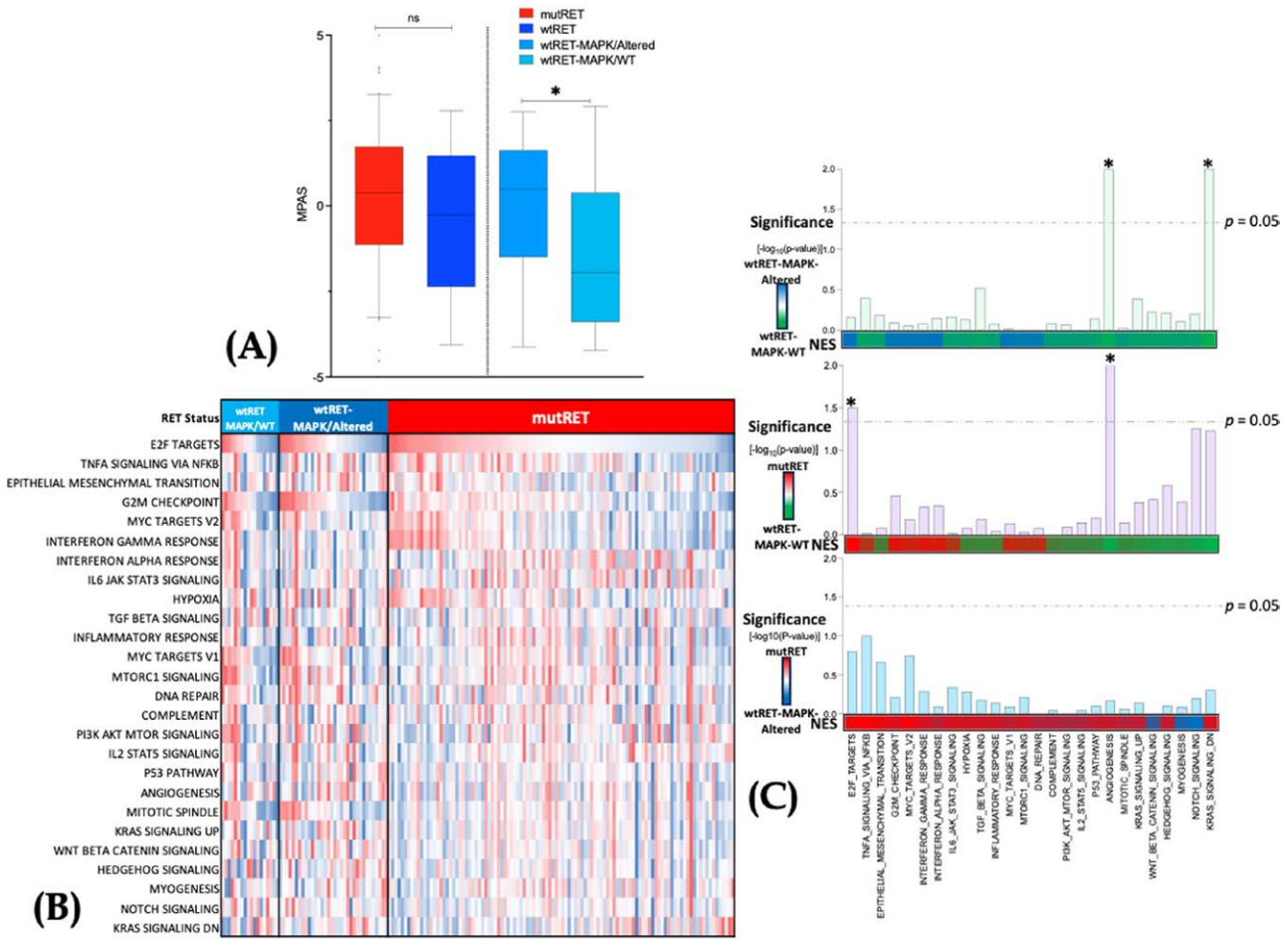

Figure 2. ssGSEA and quantification of MAPK activation calculated through expression profile of 10 MAPK-associated genes, generating MPAS. (A) MPAS comparison between mut*RET* vs wt*RET*; wt*RET*-MAPK-Altered vs wt*RET*-MAPK-WT. (B) ssGSEA comparison between mut*RET* vs wt*RET*-MAPK-Altered vs wt*RET*-MAPK-WT (ranked by the E2F_TARGETS). (C) GSEA pathway analysis: distribution of normalized enrichment score between mut*RET* vs wt*RET*-MAPK-Altered vs wt*RET*-MAPK-WT. GSEA, gene set enrichment analysis; MAPK, mitogen-activated protein kinase; MPAS, MAPK pathway activation score; mut*RET, RET* mutated; NES; ssGSEA, single-sample gene set enrichment analysis; wt*RET*, wild-type *RET*. Statistical significance was noted as *p < 0.05, **p < 0.01, ***p < 0.001.

As MPAS is a known signature for predicting MAPK activation,[23] we estimated the correlation between MPAS and the TME fractions between wt*RET* and mut*RET*. We observed significant positive correlation between MPAS signature and NK cells in both wt*RET* and mut*RET* cohort (spearman correlation: 0.23, p = 0.107; 0.26, p = 0.601; 0.30, p < 0.001). Similarly, MPAS is positively correlated with B cells (0.16, p = 0.04), macrophages M2 (0.35, p < 0.001), and Tregs (0.19, p = 0.038) in the mut*RET* cohorts (Fig. 3A). There may be a positive correlation between MPAS and CD4 T cells in wt*RET*-MAPK-WT (spearman: 0.35, p = 0.186)—although not a statistically significant one in our study and the correlation with CD8 T cells were relatively low across all samples (Fig. 3A). We also observed that predictive markers of immunotherapy (TMB-H and PD-L1) are clustered in wt*RET*-MAPK-WT with lower MPAS (Fig. 3A).

Finally, we examined the association of wt*RET* MTC with putative or known predictors of response to immunotherapy. These predictors include PD-L1 expression, TMB, and the ratio of dMMR/MSI-H.[26-28] We also characterized the immune signature and interferon gamma signature score from our transcriptomic data. Our data revealed higher interferon gamma score (Fig. 3C) in wt*RET*-MAPK-WT cohorts compared with mut*RET* cohorts (median: −0.46 vs −0.59, p = 0.049), although no difference was observed between wt*RET*-MAPK-Altered compared with mut*RET* (median: −0.51 vs −0.59, p = 0.105). There was no higher prevalence of PD-L1 expression and dMMR/MSI-H in wt*RET*-MAPK-WT and wt*RET*-MAPK-Altered compared with mut*RET* (PD-L1: 12.5% vs 9.09% vs 7%, p = 0.722; dMMR/MSI-H: 5.56% vs 2.94% vs 0.9%, p = 0.247), although the

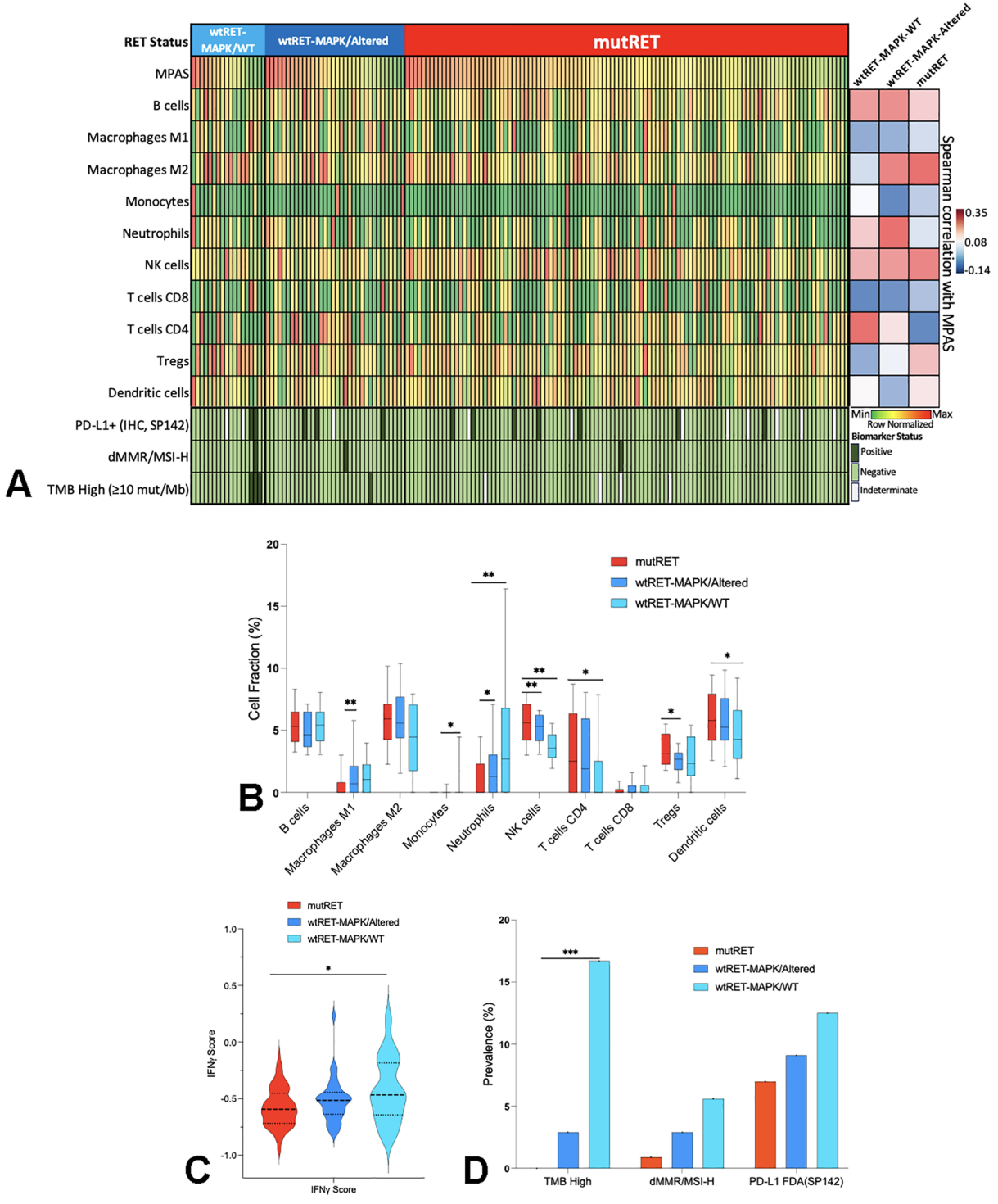

Figure 3. Tumor microenvironment and predictors of response to immunotherapy. (A) Heatmap showing the immune/stromal cell population across MTC. (B) Comparison between mut*RET* and wt*RET* shows NK cells and Tregs are significantly higher in mut*RET*, whereas macrophages M1 and neutrophils are significantly higher wt*RET*. (C) Transcriptomic signature (IFN-γ) was significantly high in wt*RET*. (D) Predictors of the immune therapy—TMB, dMMR/MSI-H, and PD-L1 are higher in wt*RET* compared with mut*RET*. dMMR/MSI-H, deficient mismatch repair and microsatellite instability-high; IFN-γ, interferon gamma; MAPK, mitogen-activated protein kinase; MPAS, MAPK pathway activation score; MTC, medullary thyroid cancer; mut*RET,* *RET* mutated; NK; TMB, tumor mutational burden; Tregs; wt*RET*, wild-type *RET*. Statistical significance was noted as *p < 0.05, **p < 0.01, ***p < 0.001.

prevalence of TMB-H was significantly higher in wt*RET*-MAPK-WT tumors (16.7% vs 2.9% vs 0.0%, p = 0.0013; Figs. 3A, 3D).

Clinical impact of *RET* mutation on medullary thyroid cancer

Using real-world clinical and treatment information from insurance claims data, we explored the prognostic outcome based on the RET mutational status. We measured the OS from either the time of biopsy or start of TKIs. For this analysis, data for 156 of 160 patients were available. We demonstrated no difference in survival among the 3 groups (mut*RET* vs wt*RET*-MAPK-Altered, 35.5 vs 27.9 months, HR 1.02, 95% CI 0.64 to 1.62, p = 0.94; mut*RET* vs wt*RET*-MAPK-WT, 35.5 vs 34.6 months, HR 1.20, 95% CI 0.65 to 2.21, p = 0.56). We also considered patients with MTC treated with selected TKI (carbozantinib, selpercatinib, or vandetanib, 64) and observed no statistically significant difference in OS from start of TKIs (mut*RET* vs wtRET-MAPK-Altered, 25.4 vs 17.3 months, HR 1.15, 95% CI 0.45 to 2.97, p = 0.77; mut*RET* vs wtRET-MAPK-WT, 25.4 vs 28 months, HR 0.54, 95% CI 0.16 to 1.77, p = 0.31).

DISCUSSION

The decreased cost of high-throughput NGS technology during the past decade has led to increased accessibility to these techniques, which has in turn enabled the large-scale sequencing of transcriptomes and genomes of various cancers, including MTC.[18,29,30] These studies have provided a wealth of information on known and novel mutations in important genes associated with cancer development and prognosis, such as *RET* mutations in sporadic MTC—for example, it has been reported that somatic *RET* mutations are associated with increased risks for pT3/T4, nodal involvement, distant metastasis, and tumor recurrence in sporadic MTC.[31] In addition, the OS rate for patients with mut*RET* treated with a multi-TKI, such as cabozantinib, has significantly increased from around 19 to more than 44 months in patients with *RET* M918T mutations.[1] In patients whose tumors progressed on either vandetinib or cabozantinib, treatment with selpercatenib, a more specific *RET* antagonist, was associated with a 73% response rate (95% CI 62 to 82), and 1-year progression-free survival of 92% (95% CI 82 to 97).[4] Cabozantinib and vandetinib are approved for all advanced MTC, but these agents appear to be less effective against wt*RET*-mutated MTC. In a large phase 3 study examining the role of cabozantinib in patients with advanced, unresectable MTC, efficacy was established with a 28% overall response rate compared with placebo (0%; p < 0.001).[32] Progression-free survival was also improved from 4 to 11.2 months. If one examines the subgroups, the subset of 42 patients who were known to have wt*RET* tumors, the salutary was not as clear with a response rate of 25% compared with a 32% response rate for mut*RET* tumors. Similarly, for vandetinb, the overall response rate was less when no *RET* mutation was present. In a large study by Wells and colleagues,[15] the response rate for sporadic mut*RET* tumors was 51.8%; however, no response was seen in 2 patients whose sporadic tumors had no *RET* mutation. Because the number in this subgroup was small, a definitive conclusion is not possible; however, the authors noted that response rates were greater for tumors that were M918T positive compared with those that were negative. An interpretation of our study population's survival data is that survival is improved in those patients whose tumors harbor a *RET* mutation because they have responded more favorably to treatment. Taken together, these data highlight the need for more effective therapy for wt*RET*-mutated MTC.

Despite these advancements in the understanding of mut*RET* MTC, there still exists a gap in the identification of alternate genetic alterations in the context of patients with wt*RET* MTC. For example, we identified 2 patients whose tumors exhibited *NTRK2* fusions for which treatment with larotrectinib or entrectinib should be very effective.[33,34] Clarification of the molecular oncogenesis of wt*RET* MTC may then elucidate additional options for treatment. In this study, we analyzed 160 tumor samples from patients with MTC with 67.5% having *RET* mutation, mostly in the M918T position, and 32.5% were wt*RET*. Of the patients with wt*RET* MTC, most (32) had mutations in MAPK pathway–associated genes, such as *HRAS, NRAS, BRAF,* or *NF1*, which is consistent with previous reports on patients with wt*RET* MTC being driven by the MAPK pathway.[16-19] Both wt*RET* samples with MAPK alterations and mut*RET* MTC samples showed high MPAS, which is indicative of responsiveness to TKI therapies; however, wt*RET*-MAPK-WT had comparatively lower MPAS.[23]

This study found that patients with wt*RET* MTC tend to have a higher TMB and a potential for a positive response to immunotherapy in wt*RET* when compared with patients with mut*RET*. The TME further suggests that patients with wt*RET* MTC, especially those with a MAPK-WT, may be more sensitive to immunotherapy. They have a higher interferon gamma signature score, a predictive marker of response to immunotherapy. Although patients with mut*RET* MTC have more NK cells and Tregs, patients with wt*RET* MTC have

more of M1-macrophages and neutrophils. The higher neutrophils-to-lymphocytes ratio in patients with wt*RET* may indicate a higher risk of immune-related adverse events.[35,36] The efficacy of immunotherapy in treating MTC is not well documented, but clinical trials are underway to explore this issue.[37] We also found a positive correlation between MPAS signature and NK cells in both wt*RET* and mut*RET* cohorts. In mut*RET*, there was a positive correlation with B cells, macrophages M2, and Tregs, which are associated with immunosuppression in some cancers.[38,39]

GSEA showed that angiogenesis, a key characteristic of cancer, particularly in promoting invasion and metastasis, was significantly enriched in the wt*RET*-MAPK-WT cohort. Although previous research has demonstrated MTC to be a highly vascularized tumor, with overexpression of *VEGF* and *VEGFR* in MTC samples,[40-42] particularly pronounced in *RET*-mutant MTC,[43] individuals with wt*RET*-MAPK-WT status might also derive potential benefit from combination therapy involving multikinase inhibitors targeting vascular endothelial growth factor/receptors such as cabozantinib or vandefitinib in conjunction with immunotherapy.[44] In addition, there are some ongoing clinical trials to target downstream protein *ERK* with agents such as ulixertinib or ravoxertinib.[45] These trials would enroll patients with uncommon *BRAF* alterations (classes II and III), such as the one patient we had with a class II *BRAF* mutation.

Our study has some limitations to consider. First, we lack comprehensive clinical annotations, such as information regarding whether some patients received treatments that target the MAPK pathway. Similarly, we lack full clinical pathology or treatment data. It is also worth noting that our study is based on samples submitted for clinical sequencing, suggesting that the patient population likely skewed toward refractory or more advanced or metastatic stage tumors. This may have affected the mutation landscape and its progression. Nevertheless, our study stands out as a comprehensive analysis of mutations in wt*RET* MTCs, coupled with correlation to transcriptome data. Within our cohort, most of non-*RET* alterations were observed in genes associated with MAPK pathway, including *HRAS, NRAS, NF1,* or *BRAF*. Interestingly, none of these were found in *RET*-mutated MTC tumors except for one case, reaffirming previous reports of their mutual exclusivity. Overall, our study underscores the significance of using NGS techniques to identify patients with MTC who would benefit from targeted therapies. Accurate mutational status identification is crucial to maximize the effectiveness and minimize toxicity of treatments.[29,30,46]

CONCLUSIONS

A detailed characterization of molecular alterations and immune-related features distinguish wt*RET* from mut*RET* MTC. Although *RET* mutation drives MTC in the absence of other molecular alterations, we show that wt*RET* MTC frequently harbors MAPK pathway mutations, along with an increased frequency of predictive markers of immunotherapy response. These findings may indicate a potential basis for MAPK-targeted therapy, possibly in combination with immuno-oncology for selected patients with wt*RET* MTC.

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