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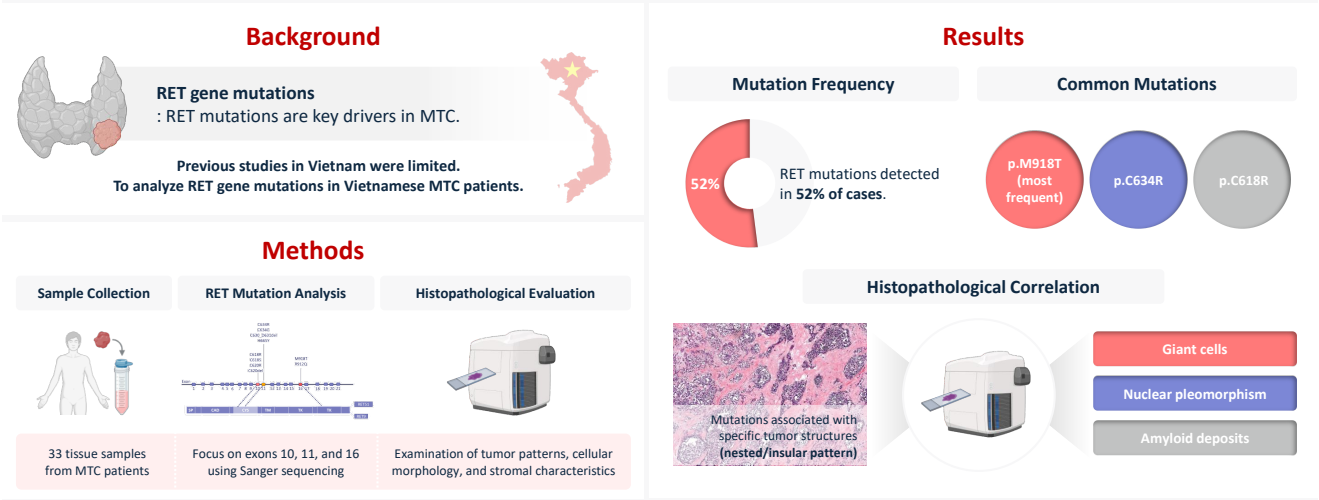
Graphical abstract



CONCLUSIONS Our results provided the first comprehensive analysis of RET mutations in Vietnamese MTC patients. The most frequent mutation is p.M918T, followed by p.C634R and p.C618R. Mutations in these three exons are linked to specific histopathological features. Information on mutational profiles of patients with MTC will further aid in development of targeted therapeutics and ensuring effective disease management.

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Background: The *RET* gene point mutation is the main molecular alteration involved in medullary thyroid carcinoma (MTC) tumorigenesis. Previous studies in Vietnam mainly consisted of case reports, with limited data on larger sample sizes. In this study, we investigated *RET* gene mutations in exons 10, 11, and 16 and analyzed clinicopathological features of a series of Vietnamese MTC patients. **Methods:** We collected 33 tissue samples from patients with MTC and analyzed *RET* mutations using the Sanger sequencing method. The relationship between hotspot *RET* mutations (exons 10, 11, 16) and clinicopathological features were investigated. **Results:** Among the 33 analyzed cases, 17 tumors (52%) harbored *RET* mutations in exon 10, 11, or 16. A total of 10 distinct genetic alterations were identified, including eight missense mutations and two short indels. Of these, seven were classified as pathogenic mutations based on previous publications, with p.M918T being the most frequent (4 cases), followed by p.C634R (3 cases) and p.C618R (3 cases). Mutations were significantly associated with specific histological patterns, such as the nested/insular pattern (p=.026), giant cells (p=.007), nuclear pleomorphism (p=.018), stippled chromatin (p=.044), and amyloid deposits (p=.024). No mutations were found in germline analyses, suggesting these were somatic alterations. **Conclusions:** Our results provided the first comprehensive analysis of *RET* mutations in Vietnamese MTC patients. The most frequent mutation was p.M918T, followed by p.C634R and p.C618R. Mutations in these three exons were linked to specific histopathological features. Information on mutational profiles of patients with MTC will further aid in the development of targeted therapeutics to ensure effective disease management.

Keywords: Thyroid neoplasms; Carcinoma, medullary; Proto-oncogene protein c-ret; Mutation

INTRODUCTION

According to the data from GLOBOCAN 2022, the global incidence of new thyroid cancer cases is 821.214, ranking seventh among all cancer types and claiming the lives of 47.507 individuals annually [1]. In Vietnam, the age-standardized incidence rate of thyroid cancer increased from 2.4 per 100,000 during 1996–2000 to 7.5 per 100,000 during 2011–2015; furthermore, the age of patients at diagnosis decreased gradually [2]. Most thyroid cancer subtypes are derived from follicular cells, except medullary thyroid carcinoma (MTC), which originates from parafollicular C cells. MTC is a well-differentiated thyroid tumor that accounts for about 5% to 10% of all thyroid carcinomas, and shows an intermediate prognostic outcome between papillary and anaplastic thyroid cancer [3,4].

The primary genetic change implicated in the development of MTC is point mutation in the *RET* gene [5]. *RET*-mutant MTC exhibits more aggressive clinical behavior, including a

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higher incidence of lymph node metastasis and distant metastasis, as well as worse survival [6-8]. Notably, patients have most benefited from the genetic screening for germline mutations of the *RET* proto-oncogene in the diagnosis, prevention, and treatment of MTC [9]. The allelic frequencies of *RET* mutations vary in different populations, and thus it is critical to ascertain population-specific mutation frequencies [10].

Takahashi et al. first described the *RET* oncogene in 1985 [11]. Situated on chromosome 10q11.2, the *RET* proto-oncogene encodes a cellular tyrosine kinase transmembrane receptor. Structurally, *RET* comprises three distinct domains: an extracellular segment at the N-terminus housing four cadherin-like regions, a cysteine-rich region housing a transmembrane domain, and a cytoplasmic domain with tyrosine kinase activity [12]. Upon binding with the ligand-co-receptor complex, *RET* undergoes dimerization and autophosphorylation on intracellular tyrosine residues, which then recruit adaptor and signaling proteins to activate multiple downstream pathways [13]. The activation of *RET* stimulates various downstream pathways involved in cell growth, proliferation, survival, and differentiation [14]. Consequently, alterations leading to the dysregulation of *RET* activity contribute to several human cancers [13].

For patients with thyroid carcinoma undergoing *RET* testing, the method typically begins by sequencing the commonly mutated *RET* cysteine codons within exons 10 and 11, along with hot spots found in exons 13 to 16. Alternatively, all *RET* exons may be sequenced from the outset [9,15]. Frequently observed somatic mutation M918T occurs in up to 40% of individuals with sporadic MTC and is linked to the aggressive nature of the disease [8,16]. In this study, none of the patients had a family history, thus we selected exons 10, 11, and 16 for analysis. MTC commonly exhibits single amino acid substitutions as well as minor insertions or deletions [15]. Since the Sanger sequencing method is the most suitable technique for analyzing single nucleotide variants and short indels, we employed the Sanger method to achieve the objectives of this study.

Research examining *RET* gene mutations in patients with MTC has been conducted in many countries worldwide. However, research in Vietnam remains limited. Therefore, this investigation aims to provide additional data on *RET* gene mutations in Vietnamese individuals, potentially contributing to diagnostic and molecular-targeted treatment applications.

MATERIALS AND METHODS

Tissue samples

We retrospectively collected primary tumors of 33 thyroid cancer patients with diagnosed MTC who had undergone thyroid resection at the Oncology Hospital (Ho Chi Minh City, Vietnam) between 2020 and 2022.

Sections were cut at 3-μm thickness and stained with hematoxylin-eosin. Experienced pathologists Q.T.P. and H.N.N. evaluated indicative regions from both cancerous and non-cancerous tissues on histopathological slides. Pathological features were classified according to the updated World Health Organization (WHO) 2022 criteria [17]. For each sample, 10 slices, each 10 μm thick, were obtained from corresponding paraffin-embedded tissue blocks for subsequent DNA extraction. Normal thyroid tissue was selected from these blocks for germline analysis.

DNA isolation

DNA extraction from formalin-fixed paraffin-embedded (FFPE) tissue blocks was carried out using the ReliaPrep FFPE gDNA Miniprep System kit (Promega, Madison, WI, USA) following the instructions of the manufacturer. The purity of the DNA samples was assessed using Nanodrop technology before polymerase chain reaction (PCR) and sequencing. Samples with insufficient DNA yield were excluded from further analysis.

PCR and Sanger sequencing

The primers of *RET* exons 10, 11, and 16 that target hot spot regions are shown in the Supplementary Table S1. All primers used in this study were newly designed. PCR was performed in 15 μL mixtures of 0.1 μM of each forward and reverse primer, 1× PCR Buffer, 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.5 U Taq Hot Start Polymerase (Takara Bio, Shiga, Japan) and 25–50 ng of genomic DNA.

PCR was denatured at 98°C for 3 minutes followed by 45 cycles of 98°C for 10 seconds, 58°C for 30 seconds and 72°C for 40 seconds, and a final elongation at 72°C for 2 minutes. PCR products were checked for size and purity using 2% agarose gel electrophoresis.

PCR products were purified enzymatically using the ExoSAP IT PCR Product Cleanup Reagent (Thermo Scientific, Waltham, MA, USA) for removal of excess primers and dNTPs before Sanger sequencing using the BigDye Terminator v3.1 Kit

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and the ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were compared to the reference sequence of the *RET* gene (GenBank accession number: NG 07489.1). CLC Main Workbench Software version 5.5 (Qiagen, Frederick, MD, USA) was utilized to analyze mutations. All detected alterations were functionally classified using the available databases (such as NCBI, COSMIC, etc.) and previous reports. Pathogenicity of variants was estimated by Polymorphism Phenotyping-2 (PolyPhen-2; Havard, Boston, MA, USA) or MutationTaster (Neurocure Cluster of Exellence/Berlin Institute of Health, Berlin, Germany).

Statistical methods

Statistical analysis was performed using STATA ver. 14.2 (Stata Corp., College Station, TX, USA). Comparisons between the two groups were conducted using the chi-square or Fisher exact test. Differences between the two groups with a significance level of $p < .05$ were considered statistically significant.

RESULTS

Characteristics of MTC patients and their clinicopathological features

Our research investigated 33 MTC patients. There were more females than males, with a female-to-male ratio of 1.4:1. The average age at diagnosis of MTC was 46.67 years. Of the cases studied, 85% exhibited a solitary tumor, and 88% had the tumor confined to a single lobe of the thyroid gland. The smallest tumor observed grossly measured 6 mm, whereas the largest measured 80 mm (Table 1). Among the morphological features, solid and nest/insular patterns were the most commonly observed in MTC cases (Supplementary Fig. S1). Typical cells were identified in 91% of the cases, exhibiting a round or polyhedral shape with coarsely granular chromatin (Supplementary Fig. S2). Four cases exhibited clearly defined nuclei, while only three cases displayed tumor cells with nuclear inclusions (Supplementary Fig. S3). In the stroma, 52% showed amyloid deposits; in addition, fibrosis was noted in 64% of cases and hemorrhage in 61%, with calcifications occurring in approximately 30% of the cases (Supplementary Fig. S4).

In total, 45% of MTC patients were diagnosed with lymph node metastasis. High-grade histological features included a high mitotic count (≥5 per 2 mm^2) in six cases, necrosis in three cases, and lymphovascular invasion in four cases.

Table 1. Relationship between molecular alterations and clinicopathological features

Variable	Wild-type tumors (n=16)	Tumor with *RET* mutations (n=17)	p-value
Sex (male/female)	10/6	4/13	.024
Age (yr), median (range)	45 (30–65)	50 (35–70)	.112
Tumor size (mm)	25 (6–80)	30 (10–75)	.145
Morphological features			
Solid pattern	13	17	.103
Nested/insular pattern	8	15	.026
Trabecular pattern	2	4	.656
Papillary pattern	4	2	.398
Follicular pattern	4	3	.688
Others	4	4	>.99
Tumor cell characteristics			
Admixtures	9	14	.141
Round or polyhedral cells	14	16	.601
Spindle cells	4	6	.708
Plasmacytoid cells	4	5	>.99
Giant cells	0	7	.007
Oncocytic cells	2	3	>.99
Small cells	1	0	.485
Nuclear pleomorphism	0	6	.018
Nuclear inclusions	2	1	.601
Stippled chromatin	12	17	.044
Nucleoli	2	2	>.99
Stromal tissue characteristics			
Necrosis	3	0	.103
Sclerosis	11	10	.554
Amyloid deposits	5	12	.024
Hemorrhage	9	11	.619
Calcification	3	7	.259
Prognostic features			
Vascular invasion	3	1	.335
Nodal metastases	9	6	.227
High mitotic count (≥5 per 2 mm^2)	4	2	.398
TNM staging			
pT1	7	5	.412
pT2	8	9	.209
pT3	1	3	.112
pN0	10	7	.354
pN1	6	10	.227
pM0	16	17	N/A
AJCC stage			
Stage I	9	5	.121
Stage II	7	10	.278
Stage III	0	2	.189

AJCC, American Joint Committee on Cancer.

Table 2. *RET* gene alterations of samples

Location	Variants	Protein changes	No. of cases	Type of variants	Classification
Exon 10	c.1852T>C	p.C618R (p.Cys618Arg)	3	Missense	Pathogenic
	c.1853G>C	p.C618S (p.Cys618Ser)	1	Missense	Pathogenic
	c.1858T>C	p.C620R (p.Cys620Arg)	1	Missense	Pathogenic
	c.1858_1860del	p.C620del (p.Cys620del)	1	Deletion	Disease causing (predicted score by MutationTaster: 0.99)
Exon 11	c.1900T>C	p.C634R (p.Cys634Arg)	3	Missense	Pathogenic
	c.1900T>G	p.C634G (p.Cys634Gly)	1	Missense	Pathogenic
	c.1887_1893delinsA	p.C630_D631del (p.Cys630_Asp631del)	1	Deletion	Polymorphysm (predicted score by MutationTaster: 0.58)
	c.1993C>T	p.H665Y (p.His665Tyr)	1	Missense	Posibly Damaging (predicted score by PolyPhen-2: 0.89)
Exon 16	c.2753T>C	p.M918T (p.Met918Thr)	4	Missense	Pathogenic
	c.2735G>A	p.R912Q (p.Arg912Gln)	1	Missense	Pathogenic

The landscape of *RET* gene mutations

The prevalence of *RET* gene alterations in the hot spot region was approximately 52% in our population. As shown in Table 2 and Fig. 1, among the 33 analyzed samples, 10 alterations were identified across exons 10, 11, and 16, with frequencies of six (18%), six (18%), and five (15%), respectively. All alterations indicate heterozygous status, and no germline mutations were detected in normal tissue among these cases, suggesting that these variants were somatic mutations. The p.M918T mutation had the highest frequency with four identified cases, followed by p.C634R and p.C618R mutations with three cases each. There were two mutations at the hot spot codon p.C618 (TGC>CGC, TGC>TCC) in exon 10 and two mutations at the hot spot codon p.C634 (TGC>CGC, TGC>GGC) in exon 11.

Alongside the eight single nucleotide variants (p.C618R, p.C618S, p.C620R, p.C634R, p.C634G, p.H665Y, p.M918T and p.R912Q), two short in-frame insertions/deletions were observed: a 2-amino acid deletion in exon 11 (p.C630_D631del) and a 1-amino acid deletion in exon 16 (p.C620del). Out of the detected alterations, seven were pathogenic mutations according to prior publications. Two alterations were categorized as either 'disease-causing' or 'possibly damaging,' with PolyPhen-2 and MutationTaster assigning scores of 0.89 and 0.99, respectively. Additionally, one alteration was classified as a polymorphic variant, with a score of 0.58 on MutationTaster (Table 2).

Our data showed that there are relationships between the detected mutations and histopathological features, including nested or insular pattern, giant cells, nuclear pleomorphism, stippled chromatin, and amyloid deposit (Table 1). The number of cases with detected mutations was also higher in females (p=.024). Other clinicopathological characteristics did not show any statistically significant associations with the presence or absence of mutations.

DISCUSSION

In this study, the absence of a family history along with classification of the identified variants as somatic indicated that these MTC cases harbored somatic gene alterations. With an average age of 46.67±13.93 years, this population is consistent with the age range reported by the American Thyroid Association, which notes that sporadic MTC typically presents later, often between the fourth and sixth decades of life, in contrast to hereditary MTC, which tends to manifest at an earlier age [9]. The study results also indicate that most MTC cases presented as a single tumor, with the predominant histological pattern being solid. The typical cells were round or polygonal, exhibiting coarsely granular chromatin, with a low incidence of clear nuclei and nuclear inclusions, consistent with WHO classification [17]. In addition to amyloid deposition in the stroma, our study population was characterized by a high prevalence of fibrosis and hemorrhage, both exceeding 60%.

Our research described *RET* gene mutations in a series of cases of Vietnamese patients with MTC. To the best of our knowledge, only one previous study investigated *RET* gene mutations in Vietnamese patients with MTC; however, that analysis was limited to a single case. Ha et al. [18] reported that a c.2753C>T transition resulted in a missense mutation of methi-

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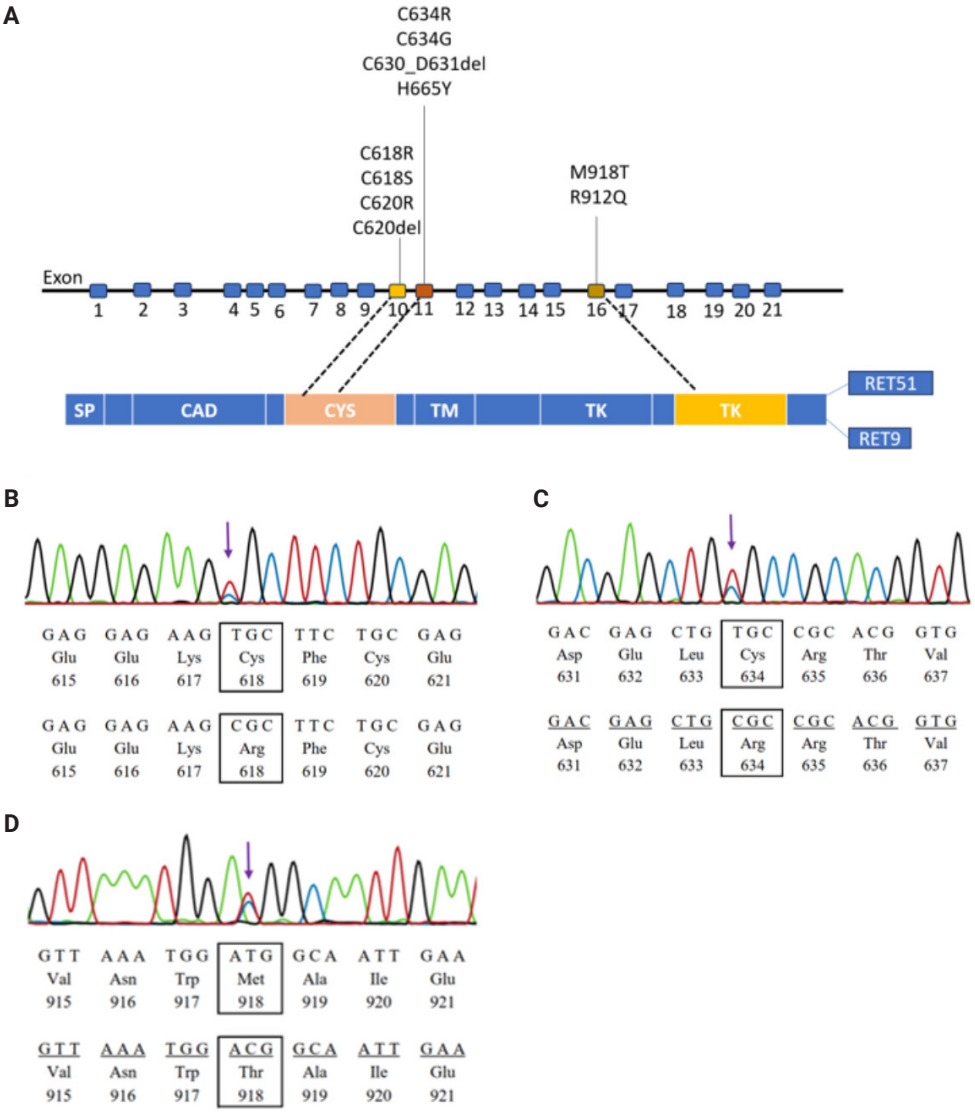

Fig. 1. The spectrum of mutations in the *RET* gene. (A) The distribution of mutations in the *RET* gene identified in this study. (B–D) The most frequently detected mutations include p.C618R in exon 10 (B), p.C634R in exon 11 (C), and p.M918T in exon 16 (D).

onine to threonine (p.M918T) in the RET protein. According to the American Thyroid Association, around half of sporadic MTC patients exhibit somatic *RET* mutations [9]. Additionally, Ciampi et al. [8] reported that somatic *RET* mutations are detected in as many as 55% of sporadic MTC patients. Our data showed mutations in exons 10, 11, and 16 in 17 cases out of a total of 33 cases of MTC with no family history, accounting for 52%.

In our study, a total of 10 different alterations in the *RET* gene were identified, including point mutations and short indels. All the point mutations were missense mutations and all the short indels were in-frame changes. In general, missense mutations with a single amino acid change were the most common ones causing loss of function of the *RET* protein in cases of MTC. Similar results have been reported in other studies [8,16].

This study detected the p.M918T mutation in exon 16 in four cases, and the p.C634R mutation in exon 11 and the p.C618R mutation in exon 10 in three cases each, suggesting that they are common in Vietnamese patients with MTC. Several publications reported that p.M918T has the highest frequency in similar studies [8,19], and cysteine mutations have been noted in sporadic MTC cases [12]. Moreover, the *RET* gene p.C634

codon was mutated in four cases with different amino acid alterations, similar to previous report [8]. The p.C634R mutation was observed in three cases. However, the p.C634 codon was mutated in four cases, including three cases of p.C634R and one additional case of p.C634G. Our data differs from a Slovakian study involving patients with MTC, where the p.C618R mutation in exon 10 was more common than p.C634R and p.M918T mutations in patients with a negative family history [20]. In this study, mutations at codon C618 were detected in four cases, more than at codon C620, which were found in two cases. These results differ from those reported by Yeganeh et al. [21], where p.C611Y and p.C620R were the most prevalent mutations in exon 10.

Asai et al. [22] and Santoro et al. [23] previously elucidated the molecular mechanisms of *RET* activation by cysteine mutations. When a non-cysteine residue replaces a cysteine residue, it releases a neighboring cysteine normally engaged in forming an intramolecular disulfide bond. This freed cysteine then creates an abnormal intermolecular covalent disulfide bond between two mutant *RET* molecules, triggering their dimerization and subsequent activation [22,23]. p.M918T mutations enhance kinase activity both as monomers and by presenting substrates for trans-autophosphorylation [23]. This effect arises from structural alterations in the activation loop of the kinase domain [24]. *RET* mutations of extracellular cysteines, which include mutations in exon 10 and exon 11, facilitate dimerization and kinase activation, whereas mutations in the *RET* kinase coding domain, including those in exon 16, drive dimerization-independent kinase activation. Thus, *RET* kinase inhibition is an attractive therapeutic target in patients with *RET* alterations. Initially, this method was accomplished through multikinase inhibitors, impacting various dysregulated pathways involving *RET* kinase. In clinical settings, employing multikinase inhibitors for advanced thyroid cancer patients yielded therapeutic benefits, although often accompanied by notable and occasionally severe side effects [15]. Nevertheless, significant advancements have emerged with the discovery of potent and specific *RET* kinase inhibitors for treating advanced thyroid cancer. While further clinical confirmation through future trials is necessary, the consistent antitumoral effectiveness and enhanced safety profile of these new compounds herald a promising era in precision oncology for *RET*-driven cancers [15]. In 2020, the U.S. Food and Drug Administration (FDA) sanctioned selpercatinib and pralsetinib for *RET*-mutated MTC necessitating systemic treatment. Drawing from these data and FDA endorsements, the National Comprehensive Cancer Network Panel advocated for both of these *RET* inhibitors as primary choices for patients with *RET*-mutant conditions [25]. Somatic genotyping for *RET* should be conducted in patients who exhibit germline wild-type status or whose germline status remains undetermined [25].

Kaserer et al. [26] highlighted that, in comparison to sporadic tumors, hereditary tumors were significantly more likely to exhibit multifocality, bilaterality, association with desmoplastic stroma, and the presence of C cell hyperplasia. Our study identified a significant association between sporadic *RET* mutations and distinct histological patterns, including a nested/insular pattern and the presence of giant cells, nuclear pleomorphisms, and amyloid deposits. To the best of our knowledge, no previous studies have reported a correlation between *RET* sporadic mutations and specific histopathological patterns. Interestingly, Verga et al. [27] reported that cutaneous lichen amyloidosis was exclusively identified in MEN2A/FMTC families carrying a *RET* pathogenic variant at codon 634. Since the number of MTC patients included in our research was relatively small, additional studies are required to better investigate the association between sporadic *RET* mutations and histological characteristics.

Our study revealed a landscape of *RET* gene mutations in exons 10, 11 and 16 in cases of MTC in Vietnamese patients. The most common mutation was p.M918T, followed by p.C634R and p.C618R. Mutations detected in these three exons are associated with histopathological features including histological patterns (nested/insular pattern), cellular features (giant cells, nuclear pleomorphism, and stippled chromatin), and stromal features (amyloid deposition). Moreover, clinical characteristics including patient sex also have a relationship with the detected mutations. The results of this study indicate that when considering *RET* gene mutations in a Vietnamese population with sporadic MTC, attention should be paid to exons 10, 11, and 16 first.

Supplementary Information

The Data Supplement is available with this article at https://doi.org/10.4132/jptm.2025.01.18.

Ethics Statement

This study received approval from the Board of Ethics in Biomedical Research of the University of Medicine and Pharmacy in Ho Chi Minh City, Vietnam, under approval number 916/

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HDDD-DHYD. The consent forms for the patients were obtained.

Availability of Data and Material
The datasets generated or analyzed during the study are available from the corresponding author on reasonable request.

Code Availability
Not applicable.

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Conflicts of Interest
The authors declare that they have no potential conflicts of interest.

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