Additive effect of RET polymorphisms on sporadic medullary thyroid carcinoma susceptibility and tumor aggressiveness

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Abstract

Objective: RET single nucleotide polymorphisms (SNPs) have been implicated in the pathogenesis and progression of medullary thyroid carcinoma (MTC). Here, we investigated the influence of multiple RET variants (G691S, L769L, S836S, and S904S) on the risk of MTC and tumor behavior.

Design and methods: One hundred and seven MTC patients and 308 cancer-unaffected control individuals were included. SNPs were analyzed using Custom TaqMan Genotyping Assays. Haplotypes based on the combination of allelic variants were inferred using a Bayesian statistical method.

Results: The minor allele frequencies in MTC patients were as follows: L769L: 28.0%, S836S: 8.9%, and G691S/S904S: 22.2%. The RET L769L and S836S SNPs were associated with increased risk of MTC (odds ratio (OR) 1.95, 95% CI: 1.2–3.1, P = 0.005 and OR 2.29, 95% CI: 1.2–4.5, P = 0.017 respectively). The adjusted OR for individuals harboring haplotypes with three or more polymorphic alleles was 3.79 (95% CI: 1.5–9.5; P = 0.004), indicating an additive effect of these variants on the risk for MTC. Among MTC patients, no significant associations were observed between RET variants and age of diagnosis or tumor size but serum calcitonin levels increased according to the number of risk alleles (P = 0.003). Remarkably, patients carrying haplotypes with three or four risk alleles had increased risk for lymph node and distant metastases at diagnosis (OR = 5.84, 95% CI: 1.1–31.2, P = 0.039). Further analysis using Kaplan–Meier model demonstrated that metastatic disease occurred earlier in individuals harboring multiple risk alleles.

Conclusion: Our results demonstrated an additive effect of RET polymorphic alleles on the estimated risk of developing aggressive MTC.

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Introduction

Medullary thyroid carcinoma (MTC), a malignant tumor originating in parafollicular C cells of the thyroid, represents 5–10% of all thyroid cancers (1, 2). About 75% of all MTCs are sporadic whereas the remaining patients have an inherited cancer syndrome known as multiple endocrine neoplasia type 2 (MEN2). Features of MEN2A include MTC, pheochromocytoma (PHEO), and hyperparathyroidism (HPT). MEN2B presents a specific phenotype that encompasses MTC, PHEO, ganglioneuromas of the digestive tract, mucosal neuromas, and/or skeletal abnormalities (1, 3). In familial MTC (FMTC), MTC is the only clinical feature (3). The rearranged during transfection (RET) proto-oncogene is the susceptibility gene for hereditary MTC. Several germline mutations of this gene have been associated with hereditary MTC. Genotype–phenotype correlation has been reported and mutations in exon 10 (codons 609, 611, 618, and 620) or exon 11 (codon 634) occur in more than 98% of MEN2A families. MEN2B is primarily associated with a single missense mutation in exon 16 (M918T), which is found in more than 90% of all reported cases (4, 5). The RET gene encodes a tyrosine kinase receptor implicated in the neural crest tissue development and differentiation.

Although much is known about hereditary MTC, the molecular mechanisms underlying sporadic MTC tumors remain to be clarified. Somatic RET point mutations in exon 16 (M918T) and gene deletions are described in 40–80% of MTC (6, 7, 8, 9). However, the mutation does not appear to be uniform among the various cell subpopulations in the same tumor or in the metastases, suggesting that MTC may be of polyclonal origin or that the mutations in the RET proto-oncogene are not initial events in MTC tumorigenesis (10, 11). In the last years, several studies have investigated whether the presence of variant sequences in the RET gene could be associated with susceptibility to development or progression of MTC (12, 13). Based on the over-representation of the RET single nucleotide polymorphisms (SNPs) G691S (exon 11, GlyGTT/SerAGT), L769L...
(exon 13, LeuCTT/LeuCTG), S836S (exon 14, SerAGC/SerAGT), and S904S (exon 15, SerTCG/SerTCG) in individuals with hereditary or sporadic MTC: a role for these genetic variants in the pathogenesis of this disease has been suggested. Nevertheless, the results on the effect of RET polymorphic variants in the predisposition to develop MTC are still conflicting. Some studies have demonstrated that the RET variant G691S is more frequent in MTC patients than in the general population (14). The G691S/S904S variants were also associated with a 1.5- to 2.5-fold risk for the development of MTC (15). Patients with MTC younger than 30 years presented a higher frequency of the RET L769L variant than those diagnosed between 31 and 45 years (16). This polymorphism was also associated with increased risk for MTC whereas patients homozygous for the minor allele of this variant were younger at the MTC diagnosis (17). Ruiz et al. (2001) (18) have demonstrated that the RET S836S variant was associated with a two- to three-fold increase in the risk of MTC in the Spanish population. More recently, the S836S variant was associated with early onset of the MTC and increased risk for metastatic disease (9). Nonetheless, several other studies failed to demonstrate any effect of RET variants on the risk for development or on the natural course of MTC (19, 20, 21). The reasons for these conflicting results are still unknown and illustrate the need for additional studies to explore the molecular mechanisms underlying MTC. Recently, two other variants of RET (IVS1–126 G>T), and (IVS8+82 A>G and 85–86 insC) have been associated with phenotypic variability in hereditary MTC patients (22). It has also been described a higher frequency of intron 14 (IVS14–24) polymorphism in MTC patients with elevated serum calcitonin concentrations (23). On the other hand, a polymorphism in exon 2, codon 45, which encodes an alanine (AlaGGG → AlaGCA), occurred at a lower frequency among the cases of MTC and, according to the authors, it could confer a protective allele against the development of MTC (15).

Carcinogenesis is a multistep process that occurs through a multifactorial interplay between many genetic and environmental factors. In this context, the effect of a single polymorphism is unlikely to be substantial in studies of complex diseases. Thus, the approach based on combining multiple polymorphisms that interact in the same pathway may amplify the effect of single variants and enhance the predictive power of polymorphism analysis for multifactorial disease (24, 25). Here, we have investigated the influence of multiple RET variants, isolated or in combination, in the estimated risk for MTC as well as in its clinical presentation. The polymorphisms G691S/S904S, L769L, and S836S were selected based on their previous association with increased risk for sporadic MTC development and aggressive clinical course.

### Materials and methods

#### Patients

Patients with a diagnosis of MTC attending the Endocrine Division at Hospital de Clínicas de Porto Alegre were invited to participate in the study. From 1997, our division has been a reference center for RET mutation testing in Brazil, and therefore, patients referred to us by other Brazilian centers were also invited to participate. All patients and/or their legal representatives provided written informed consent of the study in accordance with the institutional ethics committee.

Our study included 107 consecutive patients with MTC. The diagnosis of MTC was based on histopathological/immunohistochemistry findings and the absence of known germline RET point mutations in exons 8, 10, 11, or 13–16. Clinical and laboratory data were collected for each individual. Patients underwent a complete clinical examination, and laboratory tests were performed as described previously (9, 26). The control group was composed of 308 unrelated cancer-affected volunteers attending the blood donation facility of our Institution. A standard questionnaire was used to collect information about age, sex, skin color, and history of neoplasias. The analyses were based on the limited number of cases, and thus, to increase the statistical power, we expand the control group to 1:3 ratio.

#### Genotyping assay

RET variants G691S (rs1799939, codon 691 of exon 11, GlyGTT/SerAGT), L769L (rs1800861, codon 769 of exon 13, LeuCTT/LeuCTG: L769L(T>G)), S836S (rs1800862, codon 836 of exon 14, SerAGC/SerAGT; S836S(C>T)), and S904S (rs1800863, codon 904 of exon 15, SerTCG/SerTCG; S904S(C>G)) were analyzed. Genomic DNA was obtained from peripheral blood leukocytes by standardized procedures. Genotype analysis was performed using Human Custom TaqMan SNP Genotyping Assays 40× (Applied Biosystems, Foster City, CA, USA). Primer and probe sequences used for genotyping the RET variants were as follows: L769L (rs1800861): 5’-GGTGTGTTGACTGTTCCAG-3’ (forward primer), 5’-CTGCTGTGCTGCTATTCAG-3’ (reverse primer), VIC-5’-AGGTCTCGAGCTCA-3’, FAM-5’-AGGTCTCGAGCTCA-3’, S836S (rs1800862): 5’-GGAGAGGCCGAAAGTG-3’ (forward primer), 5’-GTGAGGCCGCAACTCTC-3’ (reverse primer), VIC-5’-CAACTCCAGCTCCCGT-3’, FAM-5’-CAACTCCAGCTCCCGT-3’, S904S (rs1800863): 5’-GCTGTGTCACGGATTTATGAA-3’ (forward primer), 5’-GGCCACCTGGCTCCCT-3’ (reverse primer), VIC-5’-CTTCAGTAGGAATCC-3’, FAM-5’-CTTCAGTAGGAATCC-3’. The two variants G691S and S904S were in linkage
disequilibrium (LD); therefore, only S904S was genotyped and the results were shown as G691S/S904S.

The reactions were conducted in a 96-well plate, in a total 5 μl reaction volume using 2 ng genomic DNA. TaqMan Genotyping Master Mix 1× (Applied Biosystems), and Custom TaqMan Genotyping Assay 1×. The plates were then positioned in a real-time PCR thermal cycler (7500 Fast Real PCR System; Applied Biosystems) and heated for 10 min at 95 °C, followed by 45 cycles of 95 °C for 15 s and 62 °C for 1 min. Fluorescence data files from each plate were analyzed using automated allele calling software (SDS 2.1; Applied Biosystems).

Statistical analysis

Results are expressed as mean ± s.d. or median (IQ 25–75) unless otherwise specified. Hardy–Weinberg equilibrium for each polymorphism was assessed by χ² tests. Baseline characteristics were compared using χ² tests or Fisher’s exact test for qualitative variables. Quantitative variables were compared between groups using Student’s t-test, ANOVA, or Kruskal–Wallis tests.

The haplotypes were constructed based on the combination of allelic variants and their frequencies were inferred using the phase 2.1 program, which implements a Bayesian statistical method (27). We also used the phase 2.1 program to compare the distributions of different RET haplotypes between MTC patients and controls through permutation analyses of 1000 random replicates (27). Between all pairs of biallelic loci, we examined widely used measures of LD, Lewontin’s D0/D1 and r² (28). Multivariate logistic regression analysis was performed to evaluate the estimated risk of MTC, using MTC as the dependent variable and age, gender, and number of RET polymorphic alleles in the haplotype as independent variables. Differences in cumulative lymph node and/or distant metastases between groups were tested by Kaplan–Meier curves, and comparisons between curves were performed using the log rank test. The Statistical Package for the Social Sciences 18.0 (PASW, Inc., Chicago, IL, USA) software was used for all analyses, and P<0.05 was considered as statistically significant.

Results

Clinical and oncological features of the subjects included in the study are summarized in Table 1. The control group comprises 308 cancer-unaffected individuals. The mean age (46.2 ± 15.8 vs 46.1 ± 9.1, P=0.91) and ethnic background (95% Caucasian) were similar between MTC patients and control group (P>0.05). However, the frequency of females was higher in MTC patients (54.6 vs 43.6%, P=0.047).

The G691S and S904S(C>G) variants were in complete LD, and therefore, to avoid redundant information, the results were grouped together and referred to as G691S/S904S(C>G) (14, 22, 29). All genotypes were analyzed using Hardy–Weinberg equilibrium (P>0.05).

Table 1 Clinical and oncological features of patients with medullary thyroid carcinoma.

<table>
<thead>
<tr>
<th></th>
<th>Total patients</th>
<th>Female (%)</th>
<th>Age at diagnosis (years)</th>
<th>Calcitonin (pg/ml)</th>
<th>CEA (ng/ml)</th>
<th>Size tumor (cm)</th>
<th>Lymph node metastases (%)</th>
<th>Distant metastases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>107</td>
<td>59 (54.6)</td>
<td>46.2 ± 15.8b</td>
<td>870.0 (154.75–2900)b</td>
<td>69.2 (11.65–166.8)b</td>
<td>3.0 (1.6–3.8)b</td>
<td>41.7</td>
<td>58.3</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± s.d. or median (IQ 25–75) unless otherwise specified. Hardy–Weinberg equilibrium for each polymorphism was assessed by χ² tests. Baseline characteristics were compared using χ² tests or Fisher’s exact test for qualitative variables. Quantitative variables were compared between groups using Student’s t-test, ANOVA, or Kruskal–Wallis tests.

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Table 2 Frequency of RET polymorphic alleles in medullary thyroid carcinoma and control groups.

<table>
<thead>
<tr>
<th>Sequence variant</th>
<th>Wild type</th>
<th>Heterozygous</th>
<th>Homozygous</th>
<th>MTC (%)</th>
<th>Controls (%)</th>
<th>Prevalence in literatureb (%)</th>
<th>OR (95% CI); Pc</th>
</tr>
</thead>
<tbody>
<tr>
<td>L769L(T&gt;G)</td>
<td>56</td>
<td>42</td>
<td>9</td>
<td>28.0</td>
<td>20.8</td>
<td>15–26</td>
<td>1.95 (1.2–3.1); 0.005</td>
</tr>
<tr>
<td>S836S(C&gt;T)</td>
<td>88</td>
<td>19</td>
<td>0</td>
<td>8.9</td>
<td>4.2</td>
<td>1.2–9.3</td>
<td>2.29 (1.2–4.5); 0.017</td>
</tr>
<tr>
<td>G691S/S904S(C&gt;G)</td>
<td>63</td>
<td>39</td>
<td>4</td>
<td>22.2</td>
<td>21.7</td>
<td>11.1–24</td>
<td>1.09 (0.6–1.7); 0.706</td>
</tr>
</tbody>
</table>

aData compared using the χ² test or Fisher’s exact test.
bReferences: 16, 17, 26, 27, 28.
cP=adjusted OR, 95% CI, and P values for the comparisons between cases and controls. The independent variables included in the multiple regression analyses were age, gender, and RET polymorphic allele.

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**Table 3** Frequency of RET haplotypes in medullary thyroid carcinoma and control groups.

<table>
<thead>
<tr>
<th>Presence/absence of Polymorphic alleles (n)</th>
<th>MTC (n=214)</th>
<th>Controls (n=616)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hpt1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hpt2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hpt3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hpt4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hpt5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hpt6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hpt7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hpt8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Hpt, haplotype; $P = 0.08$ (P value for the comparisons of haplotype frequencies between patients and controls was calculated using permutation test (1000 replications).

*a n = number of chromosomes.

**RET polymorphisms in sporadic MTC patients and controls**

The allele frequencies in MTC patients were similar to those reported in the literature (18, 19, 23, 30, 31). There were no differences in the frequency of G691S/S904S(C>G) polymorphisms between patients and controls (Table 2). Nevertheless, the L769L(T>G) polymorphic allele frequency was higher in MTC patients compared with control group (28.0 vs 20.8%, $P = 0.03$). Confirming previously published data (9), we observed a higher allelic frequency of S836S(C>G) variant in MTC patients (8.9 vs 4.2%, $P = 0.01$). When evaluating the effect of single RET polymorphisms on the risk for MTC, we found an adjusted odds ratio (OR) of 1.95 (95% CI: 1.2–3.1, $P = 0.013$) when the adjusted OR for the presence of the S836S(C>G) variant was 2.29 (95% CI: 1.2–4.5, $P = 0.017$).

**Additive effect of RET polymorphic alleles on MTC susceptibility**

Next, we evaluated whether the presence of multiple RET variants would increase the estimated risk for MTC. We used a Bayesian statistical method to estimate the frequency of different haplotypes produced by the combination of the G691S/S904S(C>G), L769L(T>G), and S836S(C>G) polymorphisms in MTC patients and controls. A total of six haplotypes were inferred in both groups (Table 3). The frequency of haplotype 3 was fivefold higher in the cases, but permutation analyses showed that the distributions of these six haplotypes were not significantly different between case and control groups ($P = 0.08$). Interestingly, none of the individuals studied presented the three polymorphic variants in the same allele (haplotype 8) or the S836S and S904S polymorphic variants in the same allele (haplotype 7). Thirteen different combinations of the six haplotypes inferred by Phase program were found in the case and control individuals (Table 4). The permutation analyses showed that the distributions of haplotypes were statistically different between case and control groups ($P = 0.035$).

We also examined LD among G691S/S904S(C>G), L769L(T>G), and S836S(C>G) polymorphisms. We did not find LD between S836S(C>G) and S904S(C>G) polymorphisms ($|D'| = 0.696; r² = 0.013$). However, the L769L(T>G) polymorphism was in partial LD with the S836S(C>G) and S904S(C>G) polymorphisms ($|D'| = 0.875; r² = 0.192$ and $|D'| = 0.871; r² = 0.084$ respectively).

In order to test the impact that multiple RET polymorphisms had on the MTC susceptibility, the individuals were grouped according to the presence of haplotypes constituted by i) no RET risk allele, ii) one or two risk alleles, or iii) three or four risk alleles. The distributions of RET risk alleles in the haplotypes were significantly different between groups (Table 5; $P = 0.02$). Interestingly, further analysis by multivariate logistic regression showed that individuals harboring three or four risk alleles in the haplotypes have increased additive effect of RET polymorphic alleles on MTC susceptibility.

**Table 4** Distribution of the various RET haplotype combinations in medullary thyroid carcinoma and control groups.

<table>
<thead>
<tr>
<th>Polymorphic alleles (n)</th>
<th>MTC, $n=107$ (%)</th>
<th>Controls, $n=308$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hpt1/Hpt1</td>
<td>None</td>
<td>26 (24.3)</td>
</tr>
<tr>
<td>Hpt1/Hpt2</td>
<td>1</td>
<td>22 (20.6)</td>
</tr>
<tr>
<td>Hpt1/Hpt3</td>
<td>1</td>
<td>4 (3.7)</td>
</tr>
<tr>
<td>Hpt1/Hpt4</td>
<td>1</td>
<td>22 (20.6)</td>
</tr>
<tr>
<td>Hpt1/Hpt5</td>
<td>2</td>
<td>4 (3.7)</td>
</tr>
<tr>
<td>Hpt2/Hpt2</td>
<td>2</td>
<td>5 (4.7)</td>
</tr>
<tr>
<td>Hpt2/Hpt4</td>
<td>2</td>
<td>9 (8.4)</td>
</tr>
<tr>
<td>Hpt2/Hpt5</td>
<td>3</td>
<td>3 (2.8)</td>
</tr>
<tr>
<td>Hpt3/Hpt4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Hpt4/Hpt4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Hpt4/Hpt5</td>
<td>3</td>
<td>7 (6.5)</td>
</tr>
<tr>
<td>Hpt5/Hpt6</td>
<td>4</td>
<td>1 (0.9)</td>
</tr>
</tbody>
</table>

Hpt, haplotype (please refer to Table 3, for haplotype description). $P$ value for the comparisons of haplotype combinations between patients and controls was 0.035 (data compared using Fisher’s exact test).
Estimated risk of MTC development (OR = 3.79; 95% CI: 1.5–9.5; \( P = 0.004 \)).

**Additive effect of RET polymorphic alleles on MTC presentation at diagnosis**

Next, we examined the additive effect of \( RET \) risk alleles (G691S/S904S(C>G), L769L(T>G), and S836S(C>T)) on clinical and oncological features of MTC patients (Table 6). No significant difference was observed in the age at diagnosis \( (P > 0.05) \) but serum calcitonin levels increased according to the number of \( RET \) risk alleles \( (P = 0.003) \). The same trend was observed for CEA levels and tumor size, although they did not reach statistical significance \( (P = 0.07 \text{ and } P = 0.06 \text{ respectively}) \). Remarkably, we found an association between number of \( RET \) risk alleles and advanced disease at diagnosis. All patients with three or four risk alleles presented lymph node metastases whereas only 50% of patients with two or less risk alleles had this feature \( (P = 0.01) \). Patients with three or four risk alleles also presented increased estimated risk for distant metastases \( (OR = 5.84, 95\% \text{ CI: } 1.1–31.2, \ P = 0.039) \). Additionally, Kaplan–Meier estimates of cumulative lymph node and distant metastases yielded distinct curves for patients with three or four risk alleles compared with those carrying one or two risk alleles \( (P = 0.001 \text{ and } P < 0.001 \text{ respectively, Fig. 1}) \), further demonstrating that metastatic disease occurred earlier in those individuals harboring multiple \( RET \) risk alleles.

### Table 6 Additive effect of \( RET \) polymorphic alleles on clinical features of patients with medullary thyroid carcinoma.

<table>
<thead>
<tr>
<th></th>
<th>None ( n = 26 )</th>
<th>One or two ( n = 70 )</th>
<th>Three or four ( n = 11 )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (years)</td>
<td>44.5 ± 18.6</td>
<td>48.3 ± 14.6</td>
<td>38.5 ± 15.4</td>
<td>0.146\a</td>
</tr>
<tr>
<td>Calcitonin (pg/ml)</td>
<td>645 (8–2331)</td>
<td>719 (236–2030)</td>
<td>7087 (3545–13 988)</td>
<td>0.003\b</td>
</tr>
<tr>
<td>CEA (ng/ml)</td>
<td>13 (7.2–14)</td>
<td>113 (19–161)</td>
<td>208 (162–255)</td>
<td>0.068\a</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>2.7 (2.0–3.7)</td>
<td>2.2 (1.5–3.5)</td>
<td>3.8 (3.4–4.4)</td>
<td>0.055\b</td>
</tr>
<tr>
<td>Lymph node metastases (%)</td>
<td>52</td>
<td>54.8</td>
<td>100</td>
<td>0.014\c</td>
</tr>
<tr>
<td>Distant metastases (%)</td>
<td>27.3</td>
<td>23</td>
<td>72.7</td>
<td>0.004\c</td>
</tr>
</tbody>
</table>

\( ^a \)Data compared using ANOVA.
\( ^b \)Data compared using Kruskal–Wallis.
\( ^c \)Data compared using \( \chi^2 \) tests.

### Discussion

This study demonstrated that the presence of multiple \( RET \) risk alleles is associated with increased estimated risk for MTC development and aggressiveness, indicating an additive effect of different \( RET \) variants in the pathogenesis of this rare malignant thyroid neoplasia. SNPs are involved in gene–environment interaction and commonly associated with a large number of sporadic cancers (32). The role of \( RET \) polymorphisms in the development or progression of MTC is still controversial. Several studies have demonstrated that \( RET \) variants may be associated with the risk of MTC as well as MTC-aggressive behavior (9, 18, 31). However, other studies failed to demonstrate such associations (19, 20, 21). Although the reasons for the conflicting results remain unsolved, differences in the genetic background of distinct population have been suggested as a potential explanation. Here, we found no differences in the frequency of G691S/S904S(C>G) polymorphisms between MTC patients and controls. However, similar to others, we observed that both L769L(T>G) and S836S(C>T) polymorphisms were associated with increased risk for MTC (9, 16, 17, 18, 31).

Combination of genetic variants that interact in the same pathway may amplify the effects of a single polymorphism and enhance the predictive power for a multifactorial disease. Thus, we wondered whether \( RET \) polymorphic variants could act synergistically in the development or progression of MTC. Our results demonstrated that this might be the case because the presence of three or more \( RET \) risk alleles was associated with a 3.79-fold increased risk for MTC development. In agreement with findings reported by Lesueur et al., we observed a partial LD between the L769L(T>G) polymorphism and S836S(C>T) and S904S(C>G) polymorphic variants. Nevertheless, contrasting with their data, we did not find LD between the S836S(C>T) and S904S(C>G) variants (33). Differences in the genetic background of distinct populations might partially explain differences in LD analysis. The LD between L769L(T>G) and S836S(C>T) polymorphisms might also explain the positive association of both the variants with MTC observed in this study and others (9, 13, 14, 15). Taken together, these observations
indicate that analysis of RET haplotypes, instead of single RET polymorphisms, may be more useful in determining the effect of RET variants on the estimated risk of MTC.

The additive effect of RET polymorphic variants becomes even more apparent in MTC disease presentation. The number of risk alleles was associated with increases in serum calcitonin levels. Moreover, we observed a nonsignificant trend toward a positive correlation between the number of risk alleles and CEA and tumor size. Notably, all MTC patients harboring three or more polymorphic alleles presented lymph node metastases at diagnosis whereas the estimated risk of distant metastases for those individuals harboring haplotypes with three or four variants was almost sixfold higher compared with those with fewer polymorphic alleles, demonstrating a cumulative effect of these variants on phenotype of disease.

The synergistic effect of polymorphisms has been described in several studies. Phillips et al. (34) showed an additive effect of polymorphisms on the IL-6, LTA, and TNFα genes and plasma fatty acid level in modulating the risk for metabolic syndrome and its components. The presence of polymorphisms in the brain-derived neurotropic factor (BDNF) and REI-silencing transcription factor (REST) genes were associated with improved general cognitive ability (35). Moreover, Sfar et al. (36) have demonstrated that gene–gene interaction of angiogenic gene polymorphisms increased the risk of prostate cancer onset and aggressiveness. Interestingly, the authors observed a significant gene–dosage effect for increasing number of potential high-risk genotypes. Compared with the reference group (low-risk genotypes), individuals with one, two, and three high-risk genotypes had increasingly elevated risks of prostate cancer. Another interesting finding of the study was that interactions between vascular endothelial growth factor (VEGF A) and thrombospondin 1 (THBS1) polymorphisms increase the risk of developing an aggressive phenotype disease. Patients carrying three high-risk genotypes showed a 20-fold increased risk of high-grade tumor (36).

The molecular mechanism by which RET polymorphisms affect the development and evolution of MTC are still not properly understood. One of the mechanisms proposed is that the polymorphism could influence the stability of the mRNA. However, quantitative studies of mRNA in MTC tumor tissues did not show a difference in RET expression in patients with or without the G691S/S904S, L769L, and S836S polymorphisms (14). Another hypothesis is that bases exchange in the DNA molecule could create a new alternative splicing site, leading to the synthesis of a truncated protein, erroneous ligand binding, micro-RNA binding, change of structure and mRNA stability as well as a number of copies, and also the change in the structure of proteins caused by interference of translation (37). Bioinformatic analysis showed differences in minimal free energy (MFE) structures in the case of exon 13. The L769L (T > G) variant reduces the energy of the wild type by 17% and the mutant Y791F by 7%, leading the authors to conclude that the L769L polymorphism reduces the MFE of small RET mRNA (17). It is also possible that this neutral variant is in LD with an as yet unknown functional variant (22, 31). Besides, in the polymorphisms that promote the substitution of amino acids, it can be assumed that the modification has a cooperative effect on the dimerization of the RET protein or forms a new phosphorylation site in the tyrosine kinase domain (29). The S836S(C > T) polymorphic variant failed to affect DNA–protein binding, transcript stability, or RNA

![Figure 1](https://www.eje-online.org)
splicing and editing (38), but it is possible that this genetic variant may create an unstable sequence upstream or downstream at germline or somatic RET mutations instead of directly participating in the tumorigenic process (31). The result of these biological interactions may influence the occurrence of somatic mutation or, alternatively, may indirectly reflect other polymorphisms that are in LD with these genotyped polymorphisms and thus contribute to the tumorigenesis of MTC.

In conclusion, our data indicate that the presence of multiple polymorphic alleles in the RET gene may increase the estimated risk for development and progression of MTC and suggest that the analysis of RET haplotypes provide higher predictive value for MTC than single polymorphism study. Nevertheless, these findings need to be confirmed on sufficiently large studies to validate or rule out a role of these variants as a cause or modifying agent in this rare disease. To the best of our knowledge, no previous studies have investigated the potential synergistic effects of RET polymorphisms on MTC.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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