The *RET* polymorphic allele S836S is associated with early metastatic disease in patients with hereditary or sporadic medullary thyroid carcinoma

Débora R Siqueira, Mírian Romitti, Andreia P da Rocha, Lucieli Ceolin, Camila Meotti, Aline Estivalet, Marcia K Puñales and Ana Luiza Maia

Abstract

The possible role of *RET* variants in modifying the natural course of medullary thyroid carcinoma (MTC) is still a matter of debate. Here, we investigate whether the *RET* variants L769L, S836S, and G691S/S904S influence disease presentation in hereditary or sporadic MTC patients. One hundred and two patients with hereditary MTC and 81 patients with sporadic MTC attending our institution were evaluated. The frequencies of *RET* polymorphisms in hereditary MTC were as follows: L769L, 17.3%; S836S, 7.95%; and S904S/G691S, 18.2%. No associations were observed between these polymorphisms and pheochromocytoma, hyperparathyroidism, lymph node, or distant metastasis. However, patients harboring the S836S variant were younger than those without this allele (17 ± 8.2 vs 28.6 ± 14.4 years, *P* = 0.01), suggesting that these patients had metastases at a young age. Accordingly, the cumulative frequency of local and/or distant metastases as estimated by Kaplan–Meier curves showed that lymph node and distant metastases occurred earlier in patients harboring the S836S variant (*P* = 0.003 and *P* = 0.026 respectively). The S836S allele frequency was higher in sporadic MTC patients than in controls (10.5 vs 3.1%, *P* = 0.01). Individuals harboring the S836S variant were younger (38.6 ± 13.3 vs 48.5 ± 16.7 years, *P* = 0.02) and showed a higher percentage of lymph node and distant metastases (*P* = 0.02 and *P* = 0.04 respectively). Kaplan–Meier estimates of lymph node and distant metastases yielded distinct curves for patients with or without the S836S allele (*P* = 0.002 and *P* = 0.001 respectively). Additional analyses using a COX regression model showed that the S836S variant was independently associated with metastatic disease (hazard ratio 2.82 (95% confidence interval 1.51–5.26), *P* = 0.001). In conclusion, the *RET* S836S variant is associated with early onset and increased risk for metastatic disease in patients with hereditary or sporadic MTC.

Endocrine-Related Cancer (2010) 17 953–963

Introduction

Medullary thyroid carcinoma (MTC), a malignant neoplasia of the parafollicular C cells of the thyroid, may occur sporadically or as part of the inherited cancer syndrome multiple endocrine neoplasia type 2 (MEN 2; Kouvaraki et al. 2005). The MEN 2 syndrome includes three clinically distinct forms: MEN 2A, MEN 2B, and familial MTC (FMTC). In patients with FMTC, only the thyroid is affected. Patients with MEN 2A develop MTC, pheochromocytoma (PHEO), and/or primary hyperparathyroidism (HPT). MEN 2B patients have MTC, PHEO, ganglioneuromas of the digestive tract, mucosal neuromas, and/or skeletal abnormalities.

The *RET* proto-oncogene is the susceptibility gene for hereditary MTC, and recent studies showed a time-dependent MTC progression, strengthening the importance of a DNA-based *RET* genotype analysis for the identification of asymptomatic gene carriers at risk of developing MTC (Machens et al. 2003, Puñales et al. 2008). Gain-of-function germline mutations in MEN 2A
and FMTC syndromes have been described in RET exons 5, 8, 10, 11, 13, 14, and 15 (Kouvaraki et al. 2005). However, the majority of MEN 2A families have mutations of one of the five conserved cysteine residues in exon 10 (codons 609, 611, 618, and 620) or exon 11 (codon 634) in the extracellular domain of RET (Eng et al. 1996, Ponder 1999). The presence of any mutation at codon 634 has been associated with the presence of PHEO and HPT. Conversely, mutations at codons 768 and 804 are thus far associated with FMTC. The reasons for these genotype–phenotype correlations have not been completely clarified yet. Although the different levels of RET activation induced by the different mutations could partially explain the phenomena, the observed clinical variability and aggressiveness in members of the same family suggest a role for genetic modifiers in the clinical course of MTC (Ponder 1999, Machens et al. 2001, Robledo et al. 2003).

The possible role of neutral RET sequence variants in modifying the MEN 2 clinical course or MEN 2-related tumors is still a matter of debate. Some studies have shown that RET single nucleotide polymorphisms (SNPs) could interfere in the disease presentation of hereditary MTC syndromes (Magalhaes et al. 2004, Rocha et al. 2007). Robledo et al. (2003) demonstrated that two of these RET variants (G691S and S904S) may modify the age at onset of MTC tumor in family members, although these findings could not be replicated in a large European population sample (Lesueur et al. 2006). It was also suggested that the L769L polymorphism might contribute to the earlier onset of MTC in a patient with a V804M mutation (Magalhaes et al. 2004). Recently, an association was described between in a patient with a V804M mutation (Magalhaes et al. 2006). It was also suggested that the L769L polymorphism might contribute to the earlier onset of MTC in a six-generation family with a G533C RET mutation (Tamanaha et al. 2009). Nevertheless, other studies failed to demonstrate any effect of RET polymorphisms on the natural course of hereditary MTC (Fernandez et al. 2006a, Lesueur et al. 2006).

The purpose of this study was to investigate whether the RET neutral variants G691S, L769L, S836S, or S904S influence the clinical presentation and disease outcome in a large cohort of individuals with MEN 2A. These polymorphisms were selected based on their previous association with the clinical course of hereditary or sporadic MTC (Robledo et al. 2003, Wiench et al. 2004, Baumgartner-Parzer et al. 2005, Lesueur et al. 2006). We observed that the S836S polymorphism was associated with younger age at diagnosis and early metastatic disease in hereditary disease. Therefore, we have decided to evaluate whether these findings would be evident in sporadic MTC.

Materials and methods

Patients

Patients with a diagnosis of hereditary MTC attending the Endocrine Division at Hospital de Clínicas de Porto Alegre were invited to participate in the study. Since 1997, our division has been a reference center for the molecular testing of RET germline mutations in Brazil, and therefore patients referred to us by other Brazilian centers for molecular investigation were also invited to participate. All patients and/or their legal guardians provided written consent in accordance with the institutional Ethics Committee.

The data collected for each individual included the clinical characteristics of family members (association of other endocrine neoplasias), the type of RET mutations, and information on atypical features noted, such as Hirschsprung’s disease (HIRS) or cutaneous lichen amyloidosis (CLA). Patients underwent a complete clinical examination, laboratory tests (levels of basal calcitonin (Until December 2003, Calcitonin IRMA-DSL7700, Diagnostic Systems Laboratories, Inc., Webster, TX, USA, reference range <10 pg/ml and, after January 2004, Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA, USA; reference value (VR) male <12.0 pg/ml and female <6.0 pg/ml)), plasma parathyroid hormone (PTH; Immulite 2000 Intact PTH, Diagnostic Products), urinary fractionated metanephrines (HPLC), and, whenever indicated, diagnostic imaging investigation (cervical ultrasonography, thorax and abdominal computed tomography (CT)). Selected patients were submitted to whole-body metaiodobenzylguanidine scintigraphy to rule out PHEO and/or distant metastasis.

Our initial sample comprised 102 patients, with germ line mutations of the RET proto-oncogene and/or immunohistochemistry diagnosis of MTC, who were identified by genetic screening at our institution, belonging to 17 unrelated families with MEN 2A and its variants or FMTC. Of them, 68 patients were diagnosed based on clinical evidence of disease, and 34 gene carriers were identified through genetic screening. Subjects who presented with physical signs compatible with MTC (palpable thyroid nodule and/or lymph node enlargement) at diagnosis were considered as presenting clinical disease, and individuals without physical disease were considered as asymptomatic gene carriers. Fourteen of these patients were excluded, either because they were awaiting surgery (2 patients) or not enough material was available for polymorphism analysis (11 patients). We also excluded one patient with a mutation at RET codon 768, exon 13,
Glu→Asp (E768D) due to the characteristic low-risk disease phenotype.

We also evaluated 81 patients with sporadic MTC. The diagnosis of sporadic MTC was based on the histopathological/immunohistochemistry findings and the absence of known germline RET point mutations in exons 8, 10, 11, or 13–16. The clinical and laboratorial data were collected for each individual in the sporadic group. A group of 80 health volunteers attending the blood donation facility of Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil) constituted our control group. A standard questionnaire was used to collect information about age, sex, skin color, and history of neoplasias.

Total thyroidectomy was performed in all patients with varying cervical neck dissection procedures. The diagnosis of lymph node metastasis was based on histological examination. Patients with suspicious distant metastasis (i.e. the presence of local metastases and/or serum calcitonin >150 pg/ml) underwent imaging exams (cervical, thoracic and abdomen CT (or liver magnetic resonance imaging), and bone scintigraphy). Patients with undetectable calcitonin levels were considered free of disease.

Patients with PHEO or HPT underwent specific surgery. Tumor staging was performed according to the current International Union against Cancer TNM classification (O’Sullivan & Shah 2003).

**Single nucleotide polymorphism analysis**

The following RET SNPs were selected based on their previous association with the clinical course of sporadic or hereditary MTC: G691S (codon 691 of exon 11, GlyGGT→SerAGT), L769L (codon 769 of exon 13, LeuCTT→LeuCTG), S836S (codon 836 of exon 14, SerAGC→SerAGT), and S904S (codon 904 of exon 15, SerTCC→SerTGC). For genotyping, genomic DNA was prepared from peripheral blood leukocytes by standard procedures, and the fragments covering the RET variants were amplified using the PCR primers and conditions previously described (Punales et al. 2003). Genotyping was performed using either restriction fragment length polymorphism (RFLP) or direct sequencing. For RFLP analysis, an aliquot of PCR product was digested with the appropriate restriction enzyme and analyzed as previously described (Punales et al. 2003). For sequencing, PCR products were purified using the GFX PCR DNA purification kit (GE Healthcare, Buckinghamshire, UK) and submitted to direct sequencing using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA).

**Somatic M918T mutation analysis**

For sporadic patients, we analyzed the frequency of somatic M918T mutations. The MTC samples were material paraffin-embedded formalin-fixed tissue blocks. DNA was extracted using the Magnesil Genomic Fixed Tissue System (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Exon 16 was amplified by PCR using 100–300 ng/μl of DNA in a reaction mix (25 μl) containing 20 mM Tris–HCl, pH 8.0, 50 mM KCl, 2 mM MgCl2, 0.2 mM dNTPs, 0.2 mM of each primer, and 1.25 U Platinum Taq DNA Polymerase (Invitrogen Life Technology, Carlsbad, CA, USA). The running profile of the amplification and RFLP analysis were similar to those described for genomic DNA (Punales et al. 2003).

**Statistical analysis**

Results are expressed as mean±s.d. or median and interquartile intervals unless otherwise specified. Baseline characteristics were compared using the χ²-test or Fisher’s exact test for qualitative variables, or the Student’s t-test or Mann–Whitney’s U test for quantitative variables. Hardy–Weinberg equilibrium for each polymorphism was assessed by the Fisher’s exact test. The differences in cumulative lymph node and/or distant metastasis between groups were tested by Kaplan–Meier curves; comparisons between curves were performed using the log rank test. We performed a Cox regression model to investigate the effect of several variables upon the time of a specified event: the presence of metastasis. The Statistical Package for the Social Sciences 15.0 (SPSS Inc., Chicago, IL, USA) was used, and P<0.05 was considered as statistically significant.

**Results**

**Frequency of RET polymorphisms in MEN 2A patients**

Table 1 shows the clinical and molecular data of the families with MEN 2A. Of the 17 independent families with hereditary MTC analyzed, 13 were classified as MEN 2A, 3 classified as MEN 2A associated with CLA, 1 associated with HIRS, and 1 classified as FMTC. All but four MEN 2A/FMTC kindreds had a mutation at RET codon 634 in exon 11, the most prevalent mutation accounting for 89% of cases. The identified mutations were as follows: Cys→Tyr (C634Y, 65.9%), Cys→Arg (C634R, 15.4%), and Cys→Trp (C634W 7.7%).

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The allele frequencies of the \textit{RET} polymorphisms are shown in Table 2. The observed SNP frequencies were similar to those reported in the literature (Elisei et al. 2004, Wiench et al. 2004, Baumgartner-Parzer et al. 2005, Wohllk et al. 2005, Severskaya et al. 2010). Confirming previous studies, the two variants G691S and S904S were in linkage disequilibrium, and, therefore, to avoid redundant information, the results were grouped together and referred as G691S/S904S (Robledo et al. 2003, Elisei et al. 2004, Tamanaha et al. 2009). All genotypes analyzed were in Hardy–Weinberg equilibrium ($P > 0.20$).

Clinical and oncological features of hereditary MTC patients

The clinical and oncological features of the subjects are listed in Table 3. The median basal serum calcitonin level at diagnosis was 140 (30–988.6) pg/ml. At first, we assessed whether the polymorphisms could have an effect on the age at onset of disease. Analysis of \textit{RET} variants failed to demonstrate differences in the age of diagnosis related to the presence or the absence of the $L769L$ or G691S/S904S polymorphic allele. However, patients harboring the S836S variant were significantly younger than those without this allele (17.0 ± 8.2 vs 28.6 ± 14.4 years, $P = 0.01$). There were no differences in basal serum calcitonin at diagnosis between individuals with or without S836S polymorphic allele (15 (8–344) vs 152 (36.9–1025) pg/ml, $P = 0.25$).

It is reasonable to speculate that in those patients identified through molecular diagnosis, the natural course of the disease was interrupted by this intervention, and that the age of diagnosis would be lower than that observed in those individuals in whom the disease evolved naturally. This could be a confusing factor, particularly when analyzing age at onset of the disease. Therefore, we also analyzed both groups separately to avoid selection bias. Table 4 shows the effect of \textit{RET} polymorphisms on MTC presentation in individuals diagnosed by genetic screening or clinical evidence of disease. There were no differences in age or serum calcitonin levels (37.5 (9.9–73) vs 9.5 (7.1–24.1) pg/ml, $P = 0.11$) at diagnosis between individuals with or without polymorphic alleles in the group diagnosed by genetic screening. However, the group of patients with clinical evidence of disease at diagnosis with the polymorphism S836S were younger than those without this allele (20.7 ± 8.1 vs 33.3 ± 13.1 years, $P = 0.03$). No significant differences were observed in serum calcitonin levels (540 (103.5–1800) vs 377.4 (344–410.9) pg/ml, $P = 0.73$).

None of the \textit{RET} polymorphisms were associated with the presence of PHEO, HPT, lymph node, or distant metastasis at diagnosis (Table 3). Thus, it was

Table 1 Clinical presentation and \textit{RET} germline mutations in multiple endocrine neoplasia 2A patients

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>$N$ families</th>
<th>\textit{RET} mutation</th>
<th>Affected individuals</th>
<th>CCH</th>
<th>MTC</th>
<th>PHEO</th>
<th>HPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEN 2A</td>
<td>6</td>
<td>C634Y</td>
<td>33</td>
<td>2</td>
<td>31</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>C634R</td>
<td>8</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>C634W</td>
<td>7</td>
<td>7</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>C618R</td>
<td>8</td>
<td>8</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MEN 2A + CLA</td>
<td>2</td>
<td>C634R</td>
<td>6</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>C634Y</td>
<td>25</td>
<td>25</td>
<td>3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>MEN 2A + HIRS</td>
<td>1</td>
<td>C618R</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td></td>
<td>88</td>
<td>2</td>
<td>86</td>
<td>27</td>
<td>16</td>
</tr>
</tbody>
</table>

MTC, medullary thyroid carcinoma; PHEO, pheochromocytoma; HPT, hyperparathyroidism; CCH, C-cell hyperplasia; CLA, cutaneous lichen amyloidosis; HIRS, Hirschsprung’s disease.

Table 2 Frequency of \textit{RET} polymorphisms in multiple endocrine neoplasia 2A patients ($n = 88$)

<table>
<thead>
<tr>
<th>Sequence variant</th>
<th>Wild-type</th>
<th>Heterozygous</th>
<th>Homozygous</th>
<th>Allele frequency (%)</th>
<th>Prevalence in literature$^a$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 13</td>
<td>L769L</td>
<td>58</td>
<td>26</td>
<td>4</td>
<td>17.3</td>
</tr>
<tr>
<td>Exon 14</td>
<td>S836S</td>
<td>74</td>
<td>14</td>
<td>0</td>
<td>7.95</td>
</tr>
<tr>
<td>Exon 15</td>
<td>G691S/S904S</td>
<td>59</td>
<td>26</td>
<td>3</td>
<td>18.2</td>
</tr>
</tbody>
</table>

Fisher’s exact test was used to test for Hardy–Weinberg equilibrium ($P > 0.20$).

$^a$References: Elisei et al. (2004), Wiench et al. (2004), Baumgartner-Parzer et al. (2005), Wohllk et al. (2005) and Severskaya et al. (2010).
somewhat puzzling that patients heterozygous for the S836S allele, on average 11 years younger than wild-type subjects, presented a virtually identical percentage of lymph node and distant metastases. These results suggested to us that these events occurred earlier in individuals harboring the S836S genotype. To test this hypothesis, we have used the Kaplan–Meier model. As gene dysfunction is present since birth, we assumed that the individual age at surgery would indicate the period of exposure. Kaplan–Meier estimates of cumulative lymph node and distant metastases yielded distinct curves for patients harboring the S836S allele \((P<0.003\) and \(P<0.026\) respectively, Fig. 1). Kaplan–Meier analysis of cumulative metastasis for L769L and G691S/S904S genotypes yielded similar curves (data not shown).

**RET polymorphisms in sporadic MTC patients**

Next, we evaluated the allele frequency of the S836S polymorphism in 81 patients with sporadic MTC followed at our institution. Table 5 shows the clinical characteristics of the studied patients. The median basal serum calcitonin level at diagnosis was 682 (67.7–2650) pg/ml. The control group consisted of 80 blood donor volunteers. The mean age was 48.2 \(\pm\) 10.1 years \((P=0.41)\), and the percentage of women were 59.3\% \((P=0.20)\). None had a recorded history of malignancy or endocrine disease. The ethnic background of both cases and controls was similar, with more than 95\% of Caucasians. The frequency of the S836S allele was higher in sporadic MTC patients when compared with controls \((10.5\% vs 3.2\%, P=0.01)\).

### Table 3 Clinical and oncological features of multiple endocrine neoplasia 2A patients according to the presence of the polymorphic allele

<table>
<thead>
<tr>
<th>Patients ((n))</th>
<th>Total ((88))</th>
<th>WT ((58))</th>
<th>L769L ((30))</th>
<th>WT ((74))</th>
<th>S836S ((14))</th>
<th>WT ((59))</th>
<th>G691S/ S904S ((29))</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex female (%)</td>
<td>56</td>
<td>60.3</td>
<td>56.7</td>
<td>0.91(a)</td>
<td>62.2</td>
<td>42.9</td>
<td>0.29(a)</td>
<td>55.9</td>
</tr>
<tr>
<td>Age(b)</td>
<td>27.6 (\pm) 15.8</td>
<td>28.3 (\pm) 14.9</td>
<td>25.1 (\pm) 13.1</td>
<td>0.34</td>
<td>28.6 (\pm) 14.4</td>
<td>17.0 (\pm) 8.2</td>
<td>0.01</td>
<td>28.3 (\pm) 15.4</td>
</tr>
<tr>
<td>Pheo (%)</td>
<td>27</td>
<td>20.7</td>
<td>36.7</td>
<td>0.22(a)</td>
<td>27.0</td>
<td>21.4</td>
<td>0.81(c)</td>
<td>25.4</td>
</tr>
<tr>
<td>HPT (%)</td>
<td>17</td>
<td>15.5</td>
<td>26.7</td>
<td>0.36(a)</td>
<td>18.9</td>
<td>21.4</td>
<td>0.89(c)</td>
<td>22.0</td>
</tr>
<tr>
<td>PN1(d) (%)</td>
<td>33</td>
<td>31.3</td>
<td>41.4</td>
<td>0.51(a)</td>
<td>34.8</td>
<td>36.4</td>
<td>1.0(c)</td>
<td>40.8</td>
</tr>
<tr>
<td>PM1(d) (%)</td>
<td>13</td>
<td>10.4</td>
<td>13.8</td>
<td>0.72(c)</td>
<td>12.1</td>
<td>9.1</td>
<td>1.0(c)</td>
<td>10.2</td>
</tr>
</tbody>
</table>

Pheo, pheochromocytoma; HPT, hyperparathyroidism; PN1, lymph node metastasis; PM1, distant metastasis; WT, wild-type.

\(a\)Variables were compared using the Yates’ \(\chi^2\)-test.

\(b\)Age, age at diagnosis, expressed as mean \(\pm\) s.d. Variables were compared using Student’s \(t\)-test.

\(c\)Variables were compared using the Fisher’s exact test.

\(d\)Data available for only 79 patients.

### Table 4 Clinical and oncological features of multiple endocrine neoplasia 2A patients diagnosed based on genetic screening or clinical grounds

<table>
<thead>
<tr>
<th>Patients ((n))</th>
<th>Total ((33))</th>
<th>WT ((23))</th>
<th>L769L ((10))</th>
<th>WT ((25))</th>
<th>S836S ((8))</th>
<th>WT ((25))</th>
<th>G691S/ S904S ((8))</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic screening</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex female (%)</td>
<td>52.9</td>
<td>52.2</td>
<td>60</td>
<td>0.72(a)</td>
<td>60</td>
<td>37.5</td>
<td>0.41(a)</td>
<td>52</td>
</tr>
<tr>
<td>Age(b)</td>
<td>14.0 (\pm) 7.1</td>
<td>14.4 (\pm) 3.9</td>
<td>14.4 (\pm) 10.4</td>
<td>0.98</td>
<td>14.7 (\pm) 7.3</td>
<td>13.5 (\pm) 6.5</td>
<td>0.71</td>
<td>14.4 (\pm) 7.3</td>
</tr>
<tr>
<td>n ((n))</td>
<td>55</td>
<td>35</td>
<td>20</td>
<td>49</td>
<td>6</td>
<td>34</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Clinical disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex female (%)</td>
<td>58.2</td>
<td>65.7</td>
<td>55</td>
<td>0.62(c)</td>
<td>63.3</td>
<td>50</td>
<td>0.66(a)</td>
<td>58.8</td>
</tr>
<tr>
<td>Age(b)</td>
<td>32.1 (\pm) 13.1</td>
<td>33.6 (\pm) 14.0</td>
<td>29.8 (\pm) 11.4</td>
<td>0.31</td>
<td>33.3 (\pm) 13.1</td>
<td>20.7 (\pm) 8.1</td>
<td>0.03</td>
<td>34.5 (\pm) 13.8</td>
</tr>
<tr>
<td>Pheo (%)</td>
<td>40.3</td>
<td>34.3</td>
<td>55</td>
<td>0.23(c)</td>
<td>40.8</td>
<td>50</td>
<td>0.87(a)</td>
<td>44.1</td>
</tr>
<tr>
<td>HPT (%)</td>
<td>23.9</td>
<td>22.9</td>
<td>40</td>
<td>0.29(c)</td>
<td>26.5</td>
<td>50</td>
<td>0.47(c)</td>
<td>35.3</td>
</tr>
<tr>
<td>PN1(d) (%)</td>
<td>53</td>
<td>42.9</td>
<td>60</td>
<td>0.34(a)</td>
<td>46.9</td>
<td>66.7</td>
<td>0.42(a)</td>
<td>58.8</td>
</tr>
<tr>
<td>PM1(d) (%)</td>
<td>19.4</td>
<td>14.3</td>
<td>20</td>
<td>0.71(a)</td>
<td>16.3</td>
<td>16.7</td>
<td>1.00(a)</td>
<td>14.7</td>
</tr>
</tbody>
</table>

Pheo, pheochromocytoma; HPT, hyperparathyroidism; PN1, lymph node metastasis; PM1, distant metastasis; WT, wild-type.

\(a\)Variables were compared using the Fisher’s exact test.

\(b\)Age, age at diagnosis, expressed as mean \(\pm\) s.d. Variables were compared using Student’s \(t\)-test.

\(c\)Variables were compared using the Yates’ \(\chi^2\)-test.

\(d\)Data available for only 46 patients.
but similar to that observed in the hereditary group (7.95%, \( P = 0.43 \)). Genotypes were in Hardy–Weinberg equilibrium (\( P = 0.20 \)).

Of the 81 MTC patients, 64 patients (79%) were homozygous for the wild-type allele (CC), and 17 patients (21%) were heterozygous for S836S polymorphic allele. There was no homozygosis for the S836S polymorphic allele. Individuals harboring the S836S variant were significantly younger at diagnosis than those without this allele (38.6 \( \pm \) 13.3 vs 48.5 \( \pm \) 16.7 years, \( P = 0.02 \)), had higher serum calcitonin level (4193 (1600–13 737) vs 539 (28.2–1440) pg/ml, \( P = 0.005 \), and presented a higher percentage of lymph node and distant metastases at diagnosis (81.3 vs 45.9 and 43.8 vs 17.7%, \( P = 0.02 \) and \( P = 0.04 \) respectively). Accordingly, Kaplan–Meier estimates of cumulative lymph node and distant metastases yielded distinct curves for patients with or without the S836S allele (\( P = 0.002 \) and \( P = 0.001 \) respectively, Fig. 2), further demonstrating that metastatic disease occurred earlier in those individuals harboring the S836S variant.

### Somatic M918T mutation analysis

The following step was to look for somatic \textit{RET} M918T mutation in the sporadic MTC group, since it has been shown that the presence of this missense somatic \textit{RET} mutation correlates with the presence of lymph node metastases at diagnosis (Elisei \textit{et al.} 2008) and could be a confounding factor in our analysis.

Forty paraffin-embedded MTC samples were available. Of them, we were unable to extract DNA from nine samples even after several repeated attempts. Of the 31 DNA samples available for analysis, 25 samples (80.6%) were found to have somatic M918T. Although we observed a higher frequency of the S836S allele in this subgroup of patients (11/31, 35.5%), there was no significant association between somatic M918T mutation and S836S polymorphic allele (10/25 (40%) vs 1/6 (16.7%) respectively, \( P = 0.38 \)).

### S836S polymorphism is an independent risk factor for metastatic disease in MTC patients

Because previous studies demonstrated that specific nucleotide and amino acid exchanges at codon 634 might have a direct impact on tumor aggressiveness in MEN 2A (Punales \textit{et al.} 2003, Milos \textit{et al.} 2008), we speculated whether co-segregation with a specific \textit{RET} mutation could interfere with our results.

According to the germline mutation, the 14 patients harboring the S836S polymorphic allele were distributed as following: 10 out of 62 individuals with C634Y mutation (3 kindred) and 4 out of 7 individuals with C634W mutation (1 kindred). We found that in all patients with S836S polymorphic allele, the M918T mutation was present.

<table>
<thead>
<tr>
<th>Patients (n)</th>
<th>Total (81)</th>
<th>WT (64)</th>
<th>S836S (17)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex female (%)</td>
<td>59.3</td>
<td>59.4</td>
<td>58.8</td>
<td>1.0(^a)</td>
</tr>
<tr>
<td>Age(^b)</td>
<td>27.6 ± 15.8</td>
<td>48.5 ± 16.7</td>
<td>38.6 ± 13.3</td>
<td>0.02</td>
</tr>
<tr>
<td>PN1(^c) (%)</td>
<td>50.6</td>
<td>45.9</td>
<td>81.3</td>
<td>0.02(^a)</td>
</tr>
<tr>
<td>PM1(^c) (%)</td>
<td>22.2</td>
<td>17.7</td>
<td>43.8</td>
<td>0.04(^d)</td>
</tr>
</tbody>
</table>

\( \text{PN1, lymph node metastasis; PM1, distant metastasis; WT, wild-type.} \)
\( ^a \text{Variables were compared using the Yates' } \chi^2 \text{-test.} \)
\( ^b \text{Age, at diagnosis, expressed as mean} \pm \text{s.d. Variables were compared using Student's } t \text{-test.} \)
\( ^c \text{Data available for 74 patients.} \)
\( ^d \text{Variables were compared using the Fisher's exact test.} \)
cases, the S836S variant did not co-segregate with the germline mutation and was inherited from the unaffected parent.

To further evaluate whether the effect of the S836S variant allele was associated with a specific germline mutation, we used the Cox proportional hazard survival analysis with the presence of metastasis at diagnosis as the outcome and the survival time as the age at diagnosis of MTC. To increase the statistical power of the analysis, all patients with MTC diagnosis (hereditary and sporadic groups) were included \((n=153)\). The results are shown in Table 6. The presence of the neutral \(\text{RET} \ S836S\) variant was an independent risk factor for early local or distant metastatic disease in MTC. As expected, all germline mutations except C618R were significantly associated with increased risk for early metastatic disease (Table 6). The lack of significance in the C618R association was probably due to the small number of subjects harboring this mutation (nine individuals).

**Table 6 Analysis of survival Cox regression \((n=153)\)**

<table>
<thead>
<tr>
<th>Variables</th>
<th>(b)</th>
<th>(P)</th>
<th>HR</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>S836S</td>
<td>1.04</td>
<td>0.001</td>
<td>2.82</td>
<td>(1.51–5.26)</td>
</tr>
<tr>
<td>C634W</td>
<td>1.79</td>
<td>0.007</td>
<td>6.03</td>
<td>(1.63–22.4)</td>
</tr>
<tr>
<td>C634Y</td>
<td>0.76</td>
<td>0.014</td>
<td>2.14</td>
<td>(1.17–3.93)</td>
</tr>
<tr>
<td>C634R</td>
<td>1.90</td>
<td>&lt;0.005</td>
<td>6.69</td>
<td>(2.45–18.3)</td>
</tr>
<tr>
<td>C618R</td>
<td>0.67</td>
<td>0.27</td>
<td>1.96</td>
<td>(0.59–6.51)</td>
</tr>
</tbody>
</table>

\(b\), regression coefficient; HR, hazard ratio; CI, confidence interval.

**Discussion**

In the present study, we have demonstrated that the neutral \(\text{RET} \ S836S\) polymorphism is associated with early onset and increased risk for metastatic disease at a younger age in individuals with hereditary or sporadic MTC. Patients harboring the variant allele were younger at diagnosis and presented with early local and distant metastasis, as assessed by the Kaplan–Meier model. Moreover, additional analysis using multivariate Cox regression identified this polymorphism as an independent risk factor for lymph node or distant metastasis.

Several SNPs of the \(\text{RET}\) proto-oncogene have been described in the general population as well as in patients with familial and sporadic MTC (Gimm et al. 1999, Ruiz et al. 2001, Elisei et al. 2004, Severskaya et al. 2010, Tamanaha et al. 2009). Here, we have studied the frequency of the exonic \(\text{RET}\) polymorphisms \(L769L\), \(S836S\), and \(G691S/S904S\) in 17 MEN 2A families. We did not detect significant differences related to disease phenotype or tumor stage. However, we observed that patients harboring the \(S836S\) variant allele were about 11 years younger than those with the wild-type genotype (Table 3). To rule out selection bias on the age of diagnosis due to genetic screening, patients were analyzed separately based on the presence or the absence of clinical disease at diagnosis. Of particular note, no differences in age at diagnosis in the group were diagnosed by genetic screening, but the positive association between the polymorphism \(S836S\) and earlier age at diagnosis remained in the group with clinical evidence of disease at diagnosis (Table 4). Surprisingly, despite that difference in age at diagnosis, patients with or without the polymorphic allele displayed a virtually identical percentage of lymph node and distant metastases (Table 3). Thus, we speculate that these individuals will develop metastasis at a younger age. Accordingly, Kaplan–Meier estimates of cumulative metastasis yielded distinct curves, indicating that these events occurred earlier in individuals with the \(S836S\) polymorphic allele (Fig. 1).
The presence of the S836S variant allele was also associated with younger age and a higher percentage of local and distant metastases at diagnosis in a sample of sporadic MTC patients (Table 5 and Fig. 2). A possible role of this variant in the pathogenesis of sporadic MTC has been speculated by several studies. Similar to the findings of this study, the S836S variant allele was over-represented in sporadic MTC patients from Germany, Spain, and the United States (Gimm et al. 1999, Ruiz et al. 2001), but no differences were observed between controls and French, Polish, British, Chilean, Portuguese, and Austrian patients (Wiench et al. 2001, Berard et al. 2004, Baumgartner-Parzer et al. 2005, Cebrian et al. 2005, Costa et al. 2005, Wohllk et al. 2005). The reasons for these conflicting results are still unclear. Yet, a significantly higher frequency of the S836S variant in patients with M918T somatic mutation in sporadic MTC has been reported (Gimm et al. 1999). Of interest, a kindred where the carriers of S836S developed MTC (Gimm et al. 1999) and a case of C-cell hyperplasia and primary HPT in an individual harboring the S836S polymorphism in the absence of germline RET mutations have also been reported (Brauckhoff et al. 2002).

In contrast to the large number of studies focused on the role of S836S variant in sporadic MTC, we have found only a few focused on hereditary MTC. Tamanaha et al. (2009) demonstrated that the S836S variant was over-represented in G533C carriers and non-carrier family members compared to the control population. However, they were unable to show an association between this variant and earlier age at onset in the large kindred cohort studied (Tamanaha et al. 2009). A modulating effect of the combination of polymorphic L769L with wild-type S836S on the clinical outcome of hereditary MTC has also been described (Severskaya et al. 2010). The segregation of RET V804L germline mutation and the S836S variant was reported in a Hungarian FMTC kindred comprising 80 individuals of four generations, but the co-existence of the V804L mutation and S836S polymorphism did not seem to aggravate the relatively low-risk disease phenotype (Patocs et al. 2003).

The exact mechanism by which these polymorphisms modulate MTC pathogenesis or disease presentation is still not known and is open to speculation. Even though these allelic variants do not seem to confer any transforming activity on the tyrosine kinase domain of the RET protein, cumulative studies suggest that they might modify disease susceptibility and clinical phenotype in patients with sporadic or hereditary MTC. Because it has been reported that polymorphic sequence variants can lead to the production of different amounts of mRNA, a theoretical role for the polymorphisms in the pathogenesis of MTC is that the allelic variant might influence RET mRNA expression. However, Elisei et al. (2004) did not find any significant difference in the levels of RET mRNA when comparing sporadic MTC patients with or without G691S/S904S, L769L, or S836S polymorphism. The S836S polymorphism failed to affect DNA–protein binding, transcript stability, or mRNA splicing and editing (Griseri et al. 2000), 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 (Gimm et al. 1999). Such a mechanism has been observed in the APC gene in a fraction (28%) of Ashkenazi with familial colorectal cancer where additional somatic mutations were more often found on that allele carrying a seemingly innocuous germline missense mutation predicted to result in a conservative amino acid change (Laken et al. 1997).

A novel observation in this study was the association between the S836S polymorphic allele and early metastatic disease observed in both hereditary and sporadic MTC, suggesting that this variant might interfere in tumor progression. The S836S allele did not co-segregate with the germline mutation and was aleatorily distributed among individuals with 634 mutations, the most prevalent mutation in our series. Accordingly, statistical analysis performed using the Cox proportional hazard survival model identified the S836S variant as an independent risk factor for metastatic disease in MTC (hazard ratio 2.82, Table 6). A possible confounding effect of somatic M918T mutation in the sporadic group was also addressed. The M918T mutation was present in about 80% of the samples analyzed, a frequency similar to that reported in some centers (Zedenius et al. 1995, Marsh et al. 1996, Moura et al. 2009), but that could be influenced by selection since most of the available samples were from patients followed at our institution due to advanced disease. Although we observed a much higher frequency of the S836S variant (35%) in this subgroup of patients, there was no significant association between somatic M918T mutation and the S836S polymorphic allele (P=0.383).

As previously discussed, the mechanistic explanation is speculative. An additional hypothesis is that an unknown functional variant, which may be in linkage disequilibrium with the haplotype containing the S836S variant, could possibly control the activity of the RET oncogene. An MTC-specific risk haplotype that includes the S836S and IVS1–126G>T RET
polymorphisms was previously described (Borrego et al. 2003). The IVS1–126G>T variant has also been associated with the development of sporadic MTC (Fernandez et al. 2004) and, interestingly, with earlier age at disease onset in a large Brazilian kindred cohort harboring the G533C RET mutation (Tamanaha et al. 2009). Of note, an in silico analysis revealed that this genetic variant creates a new binding site for NFAT transcription factor (nuclear factor of activated T-cells) (Borrego et al. 2003). The NFAT family of proteins has been found to be involved in cell cycle regulation, cell differentiation, cell survival, angiogenesis, tumor cell invasion, and metastasis (Lu & Huan 2007).

The RET variants L769L and G691S/S904S have also been studied as modifiers in disease presentation in both hereditary and sporadic MTC patients (Berard et al. 2004, Fernandez et al. 2006a,b, Guerrero et al. 2006). The polymorphic G691S/S904S variant of RET has been implicated as a modifier factor on the age at which MEN 2A begins (Gil et al. 2002, Robledo et al. 2003), whereas the RETL769L polymorphism has been previously implicated as having an effect in the early development of hereditary MTC in a family with a mutation in exon 14 (Magalhaes et al. 2004). However, we did not observe an association between the presence of these variants and clinical presentation of the disease in hereditary MTC. This highlights the importance of replication in different populations and might indicate differences due to genetic background or geographic areas.

Some factors unrelated to the RET polymorphisms could have interfered with the findings of the present study. Firstly, our results could represent a type 1 error. However, the S836S was significantly associated to younger age at diagnosis and early metastatic disease in patients with clinical disease in hereditary and sporadic MTC groups. These results argue against an association by chance. Secondly, due to the relatively small percentage of cases (38%) analyzed, we cannot formally rule out a confounding effect from the somatic M918T mutation in the sporadic group. Finally, this study has insufficient statistical power to exclude the possibility of an association between the L769L or G691S/S904S variants and clinical presentation of the disease in hereditary MTC (type 2 error).

In conclusion, our data indicate that the RET variant S836S is associated with increased risk for metastatic disease at a younger age in individuals with MEN 2A or sporadic MTC. If confirmed in other sample populations, these findings might have significant implications on the management of MTC, particularly on defining the ideal timing for prophylactic intervention on gene carriers.

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.

Funding
This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and Fundo de Incentivo à Pesquisa do Hospital de Clínicas de Porto Alegre (FIPE), Brazil.

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