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RET Polymorphisms and Sporadic Medullary Thyroid Carcinoma in a Portuguese Population

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The genetic basis of the sporadic form of medullary thyroid carcinoma, derived from "C" cells, is still poorly understood. Somatic mutations of *RET* proto-oncogene have been reported at a variable frequency ranging from 23% to 69%. The hypothesis that low penetrance factors, such as polymorphisms, might contribute to the phenotype of this neoplasm has been addressed in a few studies conducting to conflicting results. Herein, we studied 100 individuals (50 patients and 50 controls) aiming to compare the frequencies of G691S, L769L, S836S, and S904S *RET* polymorphisms observed in patients with respect to controls. Furthermore, meta-analysis of published studies including the present results was conducted. To test the contributory role of the above polymorphisms for the development of "C"-cell hyperplasia, we studied a group of 10 individuals selected for having a positive pentagastrin test despite the absence of a *RET* germline mutation. An over-representation of the G691S polymorphism, particularly in females, was observed in patients with respect to controls, although not reaching the level of significance. Allelic frequencies of the other three polymorphisms were not different in patients and controls. Results obtained in the admittedly small group of individuals with a positive pentagastrin test are unlikely to support a major influence of any polymorphism in the development of "C"-cell hyperplasia. The meta-analysis provided evidence for a significant association of the S691 allele with MTC (odds ratio 1.54, 95% confidence interval 1.12–2.12, $p = 0.008$) and found no significant associations for the other polymorphisms.

Key Words: Cancer susceptibility; MTC; polymorphisms; *RET*; meta-analysis.

Introduction

Medullary thyroid carcinoma (MTC) is a neoplasm of the "C" cells of the thyroid. About 75% of all MTCs are believed to be sporadic, whereas the remaining 25% are inherited cases *(1)* either as familial MTC only (FMTC) or as part of multiple endocrine neoplasia type 2 syndromes (MEN2A and 2B).

Germline gain-of-function mutations in the *RET* proto-oncogene, located on 10q11.2 and encoding a receptor with tyrosine kinase activity, have been identified in more than 90% of familial cases *(2)*. The genetic basis of sporadic cases is still poorly understood. Somatic mutations in *RET* have been reported at a variable frequency ranging from 23% to 69% *(3–11)* and loss of heterozygosity has been found at various loci *(12)*.

The possibility that low penetrance factors, such as polymorphisms, might contribute to the phenotype of MTC has been addressed in a few studies conducting to different results.

In 1994, one of us found no difference in the frequency of the polymorphism G691S between a group of 25 individuals with clinically defined sporadic or familial MTC (19 US patients and 6 Portuguese patients) and a control group of 25 unaffected Caucasian Americans *(13)*. A recent study has shown that *RET* G691S polymorphism is more frequent in MTC patients than in the normal controls *(14)*.

Over-representation of the S836S polymorphism in patients with sporadic MTC compared with control individuals was observed in two studies, one including German and US patients *(15)* and the other including Spanish patients *(16)*.

Contrasting with the above results, two other studies, enrolling French and Polish patients, failed to show any association with the variants L769L and S836S and predisposition to sporadic MTC *(17,18)*.

Herein, we evaluated the frequency of G691S, L769L, S836S, and S904S *RET* polymorphisms in germline DNA from 50 Portuguese unrelated sporadic MTC patients and 50 controls. Furthermore, 10 apparently healthy individuals, selected among family members of MTC patients for having an abnormal positive pentagastrin (PG) test, were also included because they might represent an intermediate condition of "C"-cell hyperplasia. These tests were performed prior genetic screening became a routine.

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Table 1
Allelic Frequencies of Polymorphic Variants
of *RET* Proto-oncogene in 50 MTC Cases (GI) and 50 Healthy Controls (GIII)

Exon	Nucleotide substitution	Amino acid change	Allelic frequencies GI (n = 50)	Allelic frequencies GIII (n = 50)	Statistics GI x GIII
11	c.2071G/A	G691S	0.33	0.21	p = 0.08*
13	c.2307T/G	L769L	0.16	0.18	p = 0.85*
14	c.2508C/T	S836S	0.06	0.05	p = 1**
15	c.2712C/G	S904S	0.25	0.22	p = 0.74*

*Chi-square with Yates' correction; **Fisher's exact test.

Table 2
Heterozygous and Homozygous Frequencies for the Four Polymorphisms

	G691S				L769L				S836S				S904S			
	Heterozygous (G/A)		Homozygous (A/A)		Heterozygous (T/G)		Homozygous (G/G)		Heterozygous (C/T)		Homozygous (T/T)		Heterozygous (C/G)		Homozygous (G/G)	
GI	23/50	46%	5/50	10%	12/50	24%	2/50	4%	4/50	8%	1/50	2%	17/50	34%	4/50	8%
GIII	17/50	34%	2/50	4%	14/50	28%	2/50	4%	3/50	6%	1/50	2%	18/50	36%	2/50	4%

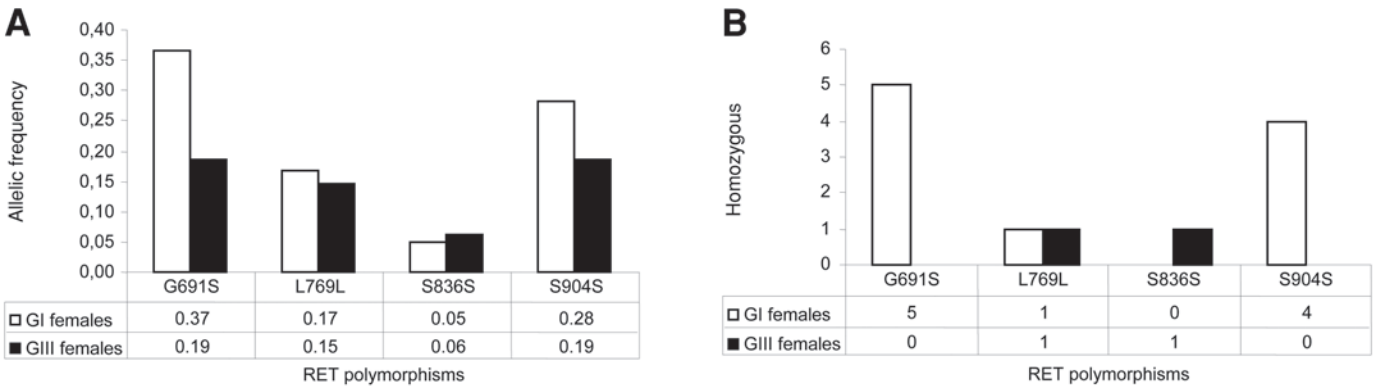

Fig. 1. Comparison between females from GI and GIII. (**A**) Allelic frequencies. (**B**) Number of homozygous females.

Results

The allelic frequencies of the polymorphisms studied are summarized in Table 1. The difference in allelic distributions between GI and normal controls was not statistically significant. The over-representation of polymorphism G691S observed in MTC patients with respect to normal controls did not reach the level of significance (χ^2 = 3.07, p = 0.08). Allelic frequencies in GII were similar to those observed in controls; however, the small number of individuals in this group does not allow a definitive conclusion. The frequency of homozygous individuals for each of the studied variants was not statistically more prevalent in the group of MTC patients as compared with the control group (Table 2).

Among patients, allelic distribution according to the gender did not disclose any significant differences between females and males. Comparison between females from GI and GIII revealed a higher frequency of the variant G691S in the former group (Fig. 1A), although not statistically significant (χ^2 = 3.353, p = 0.067). Furthermore, the homozygous status for the same polymorphism was also more frequent in females from GI than in normal females (Fig. 1B) (Fisher's exact test, p = 0.058).

Allelic distribution according to the age at onset of MTC (< 50 yr or ≥ 50 yr) did not disclose any significant differences.

Combined analysis of the present results and data from other published studies (Table 3) showed evidence for a significant association of S691 allele with MTC.

Results of the cosegregation study for the four polymorphisms are presented in Table 4. The most common association of polymorphisms, both in MTC patients and controls, was "G691S + S904S." On the contrary, polymorphisms G691S and S904S, not associated with any other polymorphisms, were observed only in MTC patients. The presence

Table 3
Summary of the Meta-analysis Results

Study	Year	MTC cases	Control	OR (95% CI)	*n* Total	*p* Value
691 (**G**GT→**A**GT)						
Gimm et al. *(15)*	1999	22/94	30/140	1.12 (0.60–2.09)	234	0.722
Elisei et al. *(14)*	2004	59/212	40/212	1.66 (1.05–2.62)	424	0.029
Present	2005	33/100	21/100	1.85 (0.98–3.50)	200	0.056
Combined (3)		114/406	91/452	1.54 (1.12–2.12)	858	0.008
769 (CT**T**→CT**G**)						
Gimm et al. *(15)*	1999	25/96	36/140	1.02 (0.56–1.84)	236	0.955
Berard et al. *(17)*	2004	41/184	45/174	0.82 (0.51–1.34)	358	0.428
Wiench et al. *(18)*	2004	63/270	49/180	0.81 (0.53–1.25)	450	0.350
Elisei et al. *(14)*	2004	46/212	51/212	0.88 (0.56–1.38)	424	0.563
Present	2005	16/100	18/100	0.87 (0.41–1.82)	200	0.707
Combined (5)		191/862	199/806	0.86 (0.69–1.09)	1668	0.210
836 (AG**C**→AG**T**)						
Gimm et al. *(15)*	1999	9/98	5/140	2.73 (0.89–8.41)	238	0.070
Ruiz et al. *(16)*	2001	6/64	18/500	2.78 (1.06–7.26)	564	0.031
Berard et al. *(17)*	2004	12/184	9/174	1.28 (0.53–3.12)	358	0.587
Wiench et al. *(18)*	2004	8/270	4/180	1.34 (0.40–4.53)	450	0.633
Elisei et al. *(14)*	2004	13/212	18/212	0.70 (0.34–1.48)	424	0.351
Present	2005	6/100	5/100	1.21 (0.36–4.11)	200	0.756
Combined (6)		54/928	59/1306	1.35 (0.91–2.01)	2234	0.140
904 (TC**C**→TC**G**)						
Gimm et al. *(15)*	1999	75/94	111/140	1.03 (0.54–1.97)	234	0.926
Elisei et al. *(14)*	2004	50/212	40/212	1.33 (0.83–2.12)	424	0.235
Present	2005	25/100	22/100	1.19 (0.61–2.28)	200	0.617
Combined (3)		150/406	173/452	1.21 (0.87–1.68)	858	0.259

Table 4
RET Polymorphisms Cosegregation Analysis

	Present study		Data published by Elisei et al. *(14)*	
RET gene polymorphisms	MTC	Controls	MTC	Controls
G691S	7/50	0/50	9/106	5/106
G691S+L769L	1/50	0/50	2/106	1/106
G691S+L769L+S836S	0/50	0/50	0/106	0/106
G691S+L769L+S904S	2/50	2/50	—	—
G691S+L769L+S836S+S904S	1/50	0/50	3/106	1/106
G691S+S836S	0/50	0/50	0/106	0/106
G691S+S904S	17/50	17/50	28/106	23/106
G691S+S836S+S904S	0/50	0/50	0/106	0/106
L769L	6/50	9/50	18/106	24/106
L769L+S836S	4/50	4/50	9/106	12/106
L769L+S836S+S904S	0/50	0/50	0/106	0/106
L769L+S904S	0/50	1/50	1/106	2/106
S836S	0/50	0/50	1/106	1/106
S836S+S904S	0/50	0/50	0/106	0/106
S904S	1/50	0/50	1/106	3/106
No polymorphism	11/50	17/50	27/106	29/106

MTC, Medullary thyroid carcinoma.

of the S836S polymorphism alone was quite rare, in both MTC patients and controls, as was its association with other polymorphisms except for the L769L polymorphism. The association "S836S + L769L" was indeed the second most common.

Discussion

Polymorphisms are allelic variants of a gene present in the general population. When they result in alterations of the protein encoded by the gene, there are no obvious phenotypic consequences. However, a few reports pointed out to the possibility that polymorphisms may act as low susceptibility factors or as modifiers of a specific disease *(19–23)*.

Different *RET* polymorphisms have been reported to be under- or over-represented in Hirschsprung disease (HSCR), which is associated with loss-of-function germline and somatic *RET* mutations *(24–27)*. Furthermore, over-representation of other *RET* polymorphisms were observed in association with congenital central hypoventilation patients either in combination or not with HSCR *(28)*.

Herein, we analyzed the frequency of four polymorphisms (G691S, L769L, S836S, and S904S) in a cohort of Portuguese MTC patients and compared results with the frequency observed in a normal population to determine whether these variants of *RET* might represent low-penetrance alleles predisposing to sporadic MTC. Selection of these targets, among other previously described polymorphisms *(13,29, 30)*, was based upon localization within exons 11, 13, 14, and 15 known as hot spots for germline mutations found in familial forms of MTC. All of the screened polymorphisms are silent mutations, except for codon 691, which results in a change in the amino acid residue from glycine to serine.

In our series, frequencies observed in normal subjects, for the four polymorphisms, were within the range of variation defined by different studies enrolling normal controls with different geographic origins *(14–17,31)*.

The frequencies of the neutral polymorphisms L769L, S836S, and S904S were similar in both MTC patients and normal controls, thus suggesting the absence of a predisposing role to the development of MTC. This is in agreement with data by Berard et al. *(17)*, Elisei et al. *(14)* and Wiench et al. *(18)* and at variance with data by others *(15,16)*.

An over-representation of the G691S polymorphism was observed in MTC patients with respect to normal controls (0.33 vs 0.21), although below the threshold of statistical significance. Similarly, an over-representation of the G691S polymorphism was seen in female patients as compared with normal females ($\chi^2 = 3.353$, $p = 0.067$). Moreover, the homozygous status for the same polymorphism was also more frequent among female patients than in normal females, although not statistically significant (Fisher's exact test, $p = 0.058$).

A significantly higher frequency of the G691S polymorphism was found between MTC patients and controls by Elisei et al. *(14)*. On the contrary, no significant difference was observed in another study by Gimm et al. *(15)*.

Regarding the cosegregation of polymorphisms, the "G691S + S904S" association was the most common but it was as frequent in MTC patients as in controls, consistent with previous data *(14,32)*, and making unlikely a predisposing role of this specific association to the development of MTC.

The hypothesis of a linkage between the over-represented G691S polymorphism and the development of "C"-cell hyperplasia was tested by analysis of data from individuals with a positive pentagastrin test despite the absence of *RET* germline mutations. Allelic frequencies were similar to those found in controls; however, the number of individuals in this group was far too small to allow definitive conclusions. Noteworthy to mention is the observation that only 2 out of 10 of these individuals were women. This observation is in agreement with a number of studies reporting peak calcitonin levels, after pentagastrin stimulation, to be higher in males than in females *(33–35)*.

Meta-analysis of published studies including the present results was performed, since association studies are capricious if based on small sample numbers. Meta-analysis showed evidence for a significant association of S691 allele with MTC (odds ratio 1.54, 95% confidence interval 1.12–2.12, $p = 0.008$) and showed no significant associations for the other polymorphisms.

Materials and Methods

We sought for polymorphisms G691S (exon 11), L769L (exon 13), S836S (exon 14), and S904S (exon 15) of *RET* in three different groups of individuals. The first group (GI) included 50 sporadic MTC patients, 30 females and 20 males, F:M 1.5:1 (mean age ± SD, 51.8 ± 15 yr, median 54). The diagnosis of MTC was based on histopathology. Sporadic MTC was defined as a lack of family history of MTC and by the absence of a *RET* germline mutation associated with any type of MEN 2. The second group (GII) consisted of 10 unrelated individuals, 2 females and 8 males, F:M 0.3:1 (mean age ± SD, 47.2 ± 18.3 yr, median 43) having in common an abnormal PG test [stimulated calcitonin (hCt) >50 and < 100 pg/mL], despite the absence of a causative germline *RET* mutation. These values were defined based on the pattern of response of a control group studied, in the past, in our laboratory and in data from the literature *(36,37)*. Calcitonin was assayed by an IRMA using a commercial kit (ELSA-hCT, CIS Biointernational, France) with a normal range of less than 10 pg/mL. The third group (GIII) represented a control group of 50 unrelated healthy blood donors, 24 females and 26 males, (mean age ± SD, 39.3 ± 11.3 yr, median 38) matched for region and race to the patients of group I.

Genomic DNA was obtained from peripheral venous blood samples and isolated by a manual method adapted from

Bowtell *(38)* and amplified by PCR using primers previously described *(39–41)*. Groups I and II were studied by direct sequencing of PCR products using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 310 Genetic Analyser, in order to exclude the presence of germline mutations and seek for polymorphisms concomitantly. The control group was screened by restriction analysis, because all the polymorphisms create or abolish a restriction site for a specific endonuclease. We used *Ban*I (or *Nla*IV), *Taq*I, *Alu*I, and *Rsa*I to study the polymorphisms G691S, L769L, S836S, and S904S, respectively. Genotypes were determined by digestion of the PCR product and electrophoresis on a polyacrylamide gel. Doubtful cases were always analyzed also by direct sequencing.

Allelic frequencies between groups were compared using standard chi-squared analysis with Yates' correction or, when appropriate, the Fisher two-tailed exact test. Statistical significance was taken as $p < 0.05$. The Comprehensive Meta-Analysis package (version 2) was used for the meta-analysis.

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