Evaluation of the role of *RET* polymorphisms/haplotypes as modifier loci for MEN 2, and analysis of the correlation with the type of *RET* mutation in a series of Spanish patients

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Abstract. Multiple endocrine neoplasia type 2 (MEN 2) is an autosomal dominant cancer syndrome, which is divided into three subtypes: MEN 2A, MEN 2B and familial medullary thyroid cancer (FMTC). Approximately 92% of MEN 2 cases are caused by mutations in exons 10, 11, 13-16 of the *RET* proto-oncogene. There exists inter- and intra-familial phenotypic variability among the MEN 2 families, even when the disease is caused by the same *RET* mutation, suggesting a role for genetic modifiers, such as polymorphisms/haplotypes. We have sought to determine the frequency and position of *RET* germline mutations in a cohort of 114 Spanish probands with any sign of MEN 2, and to search for putative modifier loci. Mutational screening of *RET* revealed 9 different mutations, present in 26 of the 114 probands (22.8%). In addition, distributions of 8 *RET* polymorphisms and the haplotypes comprising them, were studied in the context of the families positive for *RET* mutational screening, in order to evaluate them as genetic modifiers. The relationship between *RET* mutation type and presence of a polymorphism/haplotype was analyzed. The relationship between the presence of pheochromocytoma (PC) and/or hiperparathyroidism (HPT) in carriers of the same *RET* mutation, and the genotype for the specific variants was also studied. The results derived from those analyses revealed no associations of any variant/haplotype to a specific mutation or to the clinical presentation. Nevertheless, these observations do not permit us to exclude the possible role of other variants in *RET* or other related genes, in the final presentation of the disease.

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Introduction

Medullary thyroid carcinoma (MTC) is a tumour of the thyroid C cells which occurs as a part of the heritable multiple endocrine neoplasia type 2 (MEN 2, OMIM 171400) in 25% of cases, and sporadically in the remaining cases. MEN 2 is a cancer syndrome transmitted as an autosomal dominant trait, and presenting with a high degree of penetrance and variable clinical expression. MEN 2 is divided into three sub-types depending on the organs involved: MEN 2A, MEN 2B and familial MTC (FMTC). MEN 2A comprises the classic triad of MTC, pheochromocytoma (PC) in approximately 50% of cases, and hyperparathyroidism (HPT) in 15-30% of cases (1,2). The features of MEN 2B are similar to those of MEN 2A, except that the average age of tumour onset is 10 years earlier, clinically evident parathyroid disease is absent, and developmental abnormalities such as ganglioneuromatosis, medullated corneal nerves, and marfanoid habitus are present (2,3). Finally, FMTC comprises families with MTC as their only disease phenotype (2,4).

The three forms of MEN 2 are caused by specific germline mutations of the *RET* proto-oncogene, which encodes a receptor tyrosine kinase expressed in derivatives and tumours of neural crest origin (2). In >98% of MEN 2A families, the affected members have a *RET* missense mutation in one of the five cysteine codons of exon 10 (609, 611, 618, and 620) and exon 11 (634) (5,6). In each case, these changes result in replacement of a critical cysteine residue, normally involved in the intramolecular disulphide bonds that determine the tertiary structure of RET, so that the outcome is dimerisation and constitutive activation of the receptor (7-9). It has been reported that approximately 87% of MEN 2A mutations affect codon 634 and the most frequent substitution at this codon is a cysteine to arginine change (C634R), found in >50% of cases (5). In MEN 2A Spanish families high prevalence (73% of cases) was denoted of the C634Y mutation (10), which would suggest a founder effect of this mutation in the Spanish population. Many of the mutations responsible for MEN 2A have been found in >80% of FMTC families as well. FMTC has also been shown to be associated with mutations in the tyrosine kinase domain of RET, mainly

Table I. PCR conditions for *RET* mutational screening.

Exon		Primer sequences	PCR files	bp
Exon 10	Primer 5'	GCGCCCCAGGAGGCTGAGTG	95˚C-5' →[95˚C-30" →68˚C-1']X35 →72˚C-7'	186
	Primer 3'	GGTGGTGGTCCCGGCCGCC		
Exon 11	Primer 5'	TGCCAAGCCTCACACCAC	95˚C-5' →[95˚C-30" →67˚C-2']X35 →72˚C-7'	349
	Primer 3'	TCCCTCCCTGGAAGGCAG		
Exon 13	Primer 5'	GCAGGCCTCTCTGTCTGAACTT	95˚C-5' →[95˚C-30" →65˚C-1']X35 →72˚C-7'	296
	Primer 3'	GGAGAACAGGGCTGTATGGA		
Exon 14	Primer 5'	AAGACCCAAGCTGCCTGAC	95˚C-5' →[95˚C-30" →65˚C-1']X35 →72˚C-7'	343
	Primer 3'	GCTGGGTGCAGAGCCATAT		
Exon 15	Primer 5'	GTCTCACCAGGCCGCTAC	95˚C-5' →[95˚C-30" →60˚C-30" →72˚C-30"]X35 →72˚C-7'	292
	Primer 3'	ATGGTGCACCTGGGATCCCT		
Exon 16	Primer 5'	AGGGATAGGGCCTGGCCTTC	95˚C-5' →[95˚C-30" →66˚C-30" →72˚C-30"]X35 →72˚C-7'	192
	Primer 3'	TAACCTCCACCCCAAGAGAG		

affecting codons 768 (exon 13), 804 (exon 14) or 891 (exon 15) (2,6). The mechanisms by which each of these individual mutations activates RET have not been functionally demonstrated; however, they are predicted to alter either ATP or RET substrate binding (11-13). Regarding MEN 2B, it is associated primarily with a single missense mutation of codon 918 in exon 16 (M918T) which is found in >90% of all reported cases, while the mutations affecting codons 883 (exon 15) or 922 (exon 16) occur in a small number of cases (14,15). These mutations result in RET proteins with altered substrate specificity (6,16).

It has been widely demonstrated that there exist inter- and intra-familial phenotypic variability among MEN 2A families regarding the presence of PC and/or HPT, and among MEN 2A/FMTC families with respect to the age of onset, even when the disease is caused by the same germline *RET* mutation. Such variations suggest a role for genetic modifiers, which may also work through quantitative effect (17), such as the presence of additional somatic mutations or specific polymorphisms/haplotypes. The possible role of two *RET* polymorphisms, G691S (c.2071C→A, exon 11) and S904S (c.2712C→G, exon 15) has been reported on the age of onset of MEN 2A (18,19). Robledo *et al* analyzed a total of 35 unrelated MEN 2A families and found that the homozygous patients for these polymorphisms were, on average, 10 years younger at diagnosis compared with heterozygous and wild-type homozygous (19). Some other reports on FMTC families have speculated about the possible involvement of certain *RET* polymorphisms, such as L769L (c.2307T→G, exon 13) or S836 (c. 2439C→T, exon 14), in the reduced penetrance of the disease among carriers of mutations affecting the 804 codon (20,21). Results derived from a recent study revealed the over-representation or exclusive presentation of some variants within the *GFRA4*

gene (encoding a co-receptor of RET) in MEN 2 patients, when comparing with their healthy relatives, thus suggesting a putative modifying role for such polymorphisms in MEN 2 (22).

The aim of the present study was to determine the frequency and position of *RET* germline mutations in a large cohort of Spanish probands with any sign of MEN 2 syndromes, as well as to evaluate several *RET* polymorphisms/haplotypes as modifier loci. With this objective, we selected 8 *RET* variants distributed along the whole genomic sequence of the gene, located in the promoter region (-200A→G, -196C→A), intron 1 (IVS1-1463T→C, IVS1-1370C→T, IVS1-126G→T), and exons 11, 14 and 15 (G691S, S836S and S904S).

Patients and methods

Patients. In this study we analyzed a total of 114 unrelated probands from Spain ascertained and serviced by our hospital, presenting with any sign of MEN 2 syndromes. Among the 114 patients, 100 presented with MTC and they were clinically and biochemically characterized and classified as sporadic MTC (sMTC, 73 patients), FMTC (8 patients), MEN 2A (16 patients) and MEN 2B (3 patients). In one of the MEN 2A families, such phenotype co-segregates with Hirschsprung disease (HSCR, OMIM 142623), and this pedigree is described in detail in previous reports (23,24). In the remaining 14 individuals, MTC was absent but they presented with PC (10 patients), HPT (2 patients) or PC + HPT (2 patients). They were also analyzed and included in this study since PTC and/or HPT can constitute the first manifestation of the MEN 2A syndrome in some cases (25). In all the cases, diagnosis of MTC, HPT and PC was based on documented pathological examination. An informed consent was obtained from all the participants for clinical and molecular genetic

Table II. Detected mutations in the 26 *RET*+ families.

Fam. code	Exon	Codon	Base change	Amino acid change	Phenotype	*RET*+/family members studied
1	11	634	TGC/TAC	Cys/Tyr	MEN 2A	22/44
2	11	634	TGC/TAC	Cys/Tyr	MEN 2A	2/11
4	11	634	TGC/CGC	Cys/Arg	FMTC	2/4
9	11	634	TGC/TAC	Cys/Tyr	MEN 2A	8/28
11	11	634	TGC/TAC	Cys/Tyr	MEN 2A	2/4
16	15	891	TCG/GCG	Ser/Ala	FMTC	3/8
19	11	634	TGC/TAC	Cys/Tyr	MEN 2A	2/12
20	11	634	TGC/CGC	Cys/Arg	MEN 2A	2/3
23	11	634	TGC/TAC	Cys/Tyr	MEN 2A	5/15
24	10	618	TGC/CGC	Cys/Arg	FMTC	3/4
27	11	634	TGC/TAC	Cys/Tyr	MEN 2A	3/8
31	11	634	TGC/CGC	Cys/Arg	FMTC	6/11
32	10	620	TGC/TCC	Cys/Ser	MEN 2A + HSCR	2/10
35	11	634	TGC/TAC	Cys/Tyr	MEN 2A	8/24
40	11	634	TGC/TAC	Cys/Tyr	MEN 2A	2/4
44	11	634	TGC/TAC	Cys/Tyr	MEN 2A	6/11
46	10	600	CGC/CAG	Tyr/Gln	sMTC	4/7
47	16	918	ATG/ACG	Met/Thr	MEN 2B	0/7
48	11	634	TGC/CGC	Cys/Arg	MEN 2A	1/5
51	11	634	TGC/TAC	Cys/Tyr	MEN 2A	1/1
73	16	918	ATG/ACG	Met/Thr	MEN 2B	0/2
74	13	768	GAG/GAT	Glu/Asp	sMTC	0/4
91	11	634	TGC/TAC	Cys/Tyr	MEN 2A	1/6
109	10	609	TGC/TCT	Cys/Ser	MEN 2A	1/1
121	11	634	TGC/CGC	Cys/Arg	PC	0/2
137	16	918	ATG/ACG	Met/Thr	MEN 2B	0/2

The family phenotype refers to the initial diagnosis based on clinical/biochemical results and study of familial history, previous to the molecular analysis.

studies conforming to the tenets of the Declaration of Helsinki.

RET mutational screening. Genomic DNA was extracted according to standard protocols (26) from all the individuals tested. As recommended by the standard guidelines for diagnosis of MEN 2 (27), molecular analysis of exons 10, 11, 13, 14, 15 and 16 of *RET* were performed in all the 114 index patients by direct sequencing in either an ALF express semi-automated machine or a MegaBACE automated sequencer (Amersham Biosciences). Primers and PCR conditions are shown in Table I. In addition, in mutation positive cases, their relatives at risk of the disease were also screened.

Evaluation of RET variants as modifier loci for MEN 2A or FMTC. A total of 8 *RET* sequence variants were genotyped in the 259 available members of our 16 MEN 2A and 8 FMTC families. In addition, we also included the 9 available members of the families of the 2 initially considered sMTC and 1 PTC patients, who finally resulted positive for *RET* mutational screening.

The 2 polymorphisms located within the promoter region of the gene (-200A→G and -196C→A), and the intronic variants IVS1-1463T→C (rs2505533) and IVS1-1370C→T (rs2505532) were genotyped using the 'fluorescence resonance energy transfer' (FRET) technology, as previously described (28,29). The SNPs located in intron 1 (IVS1-126G→T, rs2565206), exon 11 (G691S, rs1799939), exon 14 (S836S, rs1800862) and exon 15 (S904S, rs1800863) were genotyped by differential restriction digestion with *Nla*III, *Ban*I, *Alu*I and *Rsa*I enzymes respectively, using the protocols previously described (28,30-34).

Allelic and genotypic distribution of the 8 *RET* polymorphisms and the haplotypes comprising them, were analyzed in the context of the families. The generation of haplotypes was possible due to the availability of DNA from some first-degree relatives of the patients, allowing us to reconstruct and compare the shared versus non-shared alleles. In each family, the frequencies of each variant/haplotype were calculated and compared among the affected patients and their healthy relatives.
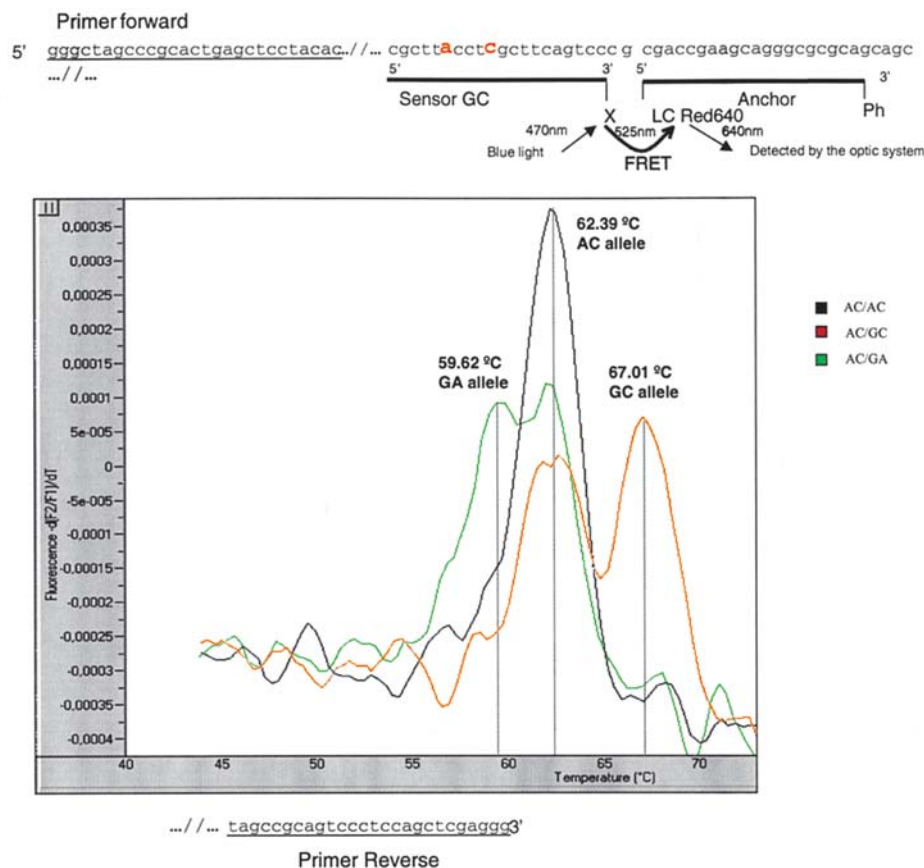
Figure 1. FRET pattern for the simultaneous detection of the *RET* variants -200A→G and -196C→A.

We have analyzed the relationship between *RET* mutation type and presence of a given *RET* polymorphism or haplotype. The relationship between the presence of PC and/or HPT in carriers of the same *RET* mutation, and the genotype for the specific variants was studied. Comparisons were performed using either Chi-square analysis with the Yate's correction, or Fisher's two-tailed exact test when appropriate. Statistical significance was set at $p < 0.05$.

Results

We screened a total of 114 unrelated probands, comprising 16 MEN 2A, 3 MEN 2B, 8 FMTC, 73 sMTC, 10 PC, 2 HPT and 2 PC + HPT patients, and found *RET* germline mutations in 26 of them (Table II). Among the familial MTC cases, only 4 clinically diagnosed FMTC families, each with 2 members affected from MTC, remain with no identified *RET* mutation. We detected 9 different types of mutations in exons 10 (R600Q, C609S, C618R, C620S), 11 (C634R, C634Y), 13 (E768D), 15 (S891A) and 16 (M918T) of *RET*, while in exon 14 no mutations were found (Table I). The most prevalent mutations were C634Y (detected in 12 of the MEN 2A families), and C634R (present in 2 MEN 2A and 2 FMTC pedigrees, as well as in a previously considered sporadic PC patient). The C620S was the cause of the MEN2A phenotype in the MEN 2A/HSCR family, and the C609S mutation was detected in another MEN 2A family. We also found two FMTC families with the C618R and the S891A mutations respectively, and another patient initially diagnosed as sporadic

MTC carried E768D. In addition, when performing mutational screening of exon 10 in one apparently sMTC patient, we detected the R600Q variant, not classically considered as a typical *RET* oncogenic mutation. Finally, the M918T was found as a *de novo* mutation in the 3 MEN 2B pedigrees.

Subsequently, we proceeded to perform molecular analysis in the relatives of the 26 *RET* mutation carriers. Among 238 relatives at risk of carrying the mutation, 86 resulted positive for this test, 43 with clinical manifestations of MEN 2 and 43 still without any sign (Table II).

The 8 *RET* variants were genotyped in the 268 available members of the 16 MEN 2A, 4 FMTC, 2 apparently sMTC, and 1 apparently sporadic PTC families which resulted positive for *RET* mutational screening. We used previously published genotyping methods, but made a modification on the test for the 2 variants of the promoter, since we employed a sensor probe different to that previously reported in Fernandez *et al* (29) (Fig. 1). We verified that the presence of any of the polymorphisms, in both homozygosity or heterozygosity, was absolutely independent of the specific pathogenic mutation of *RET* in such families. Subsequently we proceeded to construct haplotypes constituted by the combination of the alleles present at each polymorphic *RET* loci, and found 9 different specific combinations (Table III), although some of them were very infrequent. Strong co-segregation between some of the variants tested was found (Table III). Our intention was to investigate the specific *RET* allele associated with the oncogenic mutation in each family, in order to elucidate if the pedigrees sharing the same mutation might descend from

Table III. Definition of the haplotypes comprising 8 specific *RET* variants found in our families.

RET variants	0A	0B	0C	1A	1B	1C	2A	2B	2C	4
-200A→G	-	-	-	+	+	+	+	+	+	+
-196C→A	-	-	-	+	+	+	-	-	-	+
IVS1-1463T→C	-	-	-	+	+	+	+	+	+	+
IVS1-1370C→T	-	-	-	+	+	+	-	-	-	+
IVS1-126G→T	-	-	-	-	-	-	+	+	+	+
G691S	-	+	-	-	+	-	-	+	-	-
S836S	-	-	+	-	-	+	-	-	+	-
S904S	-	+	-	-	+	-	-	+	-	-

Some of the haplotypic combinations were very infrequent, such as 0B, 0C or 4. Haplotype analysis in the context of our families reveled strong cosegregation among -200A→G/IVS1-1463T→C, -196C→A/ IVS1-1370C→T and G691S/S904S.
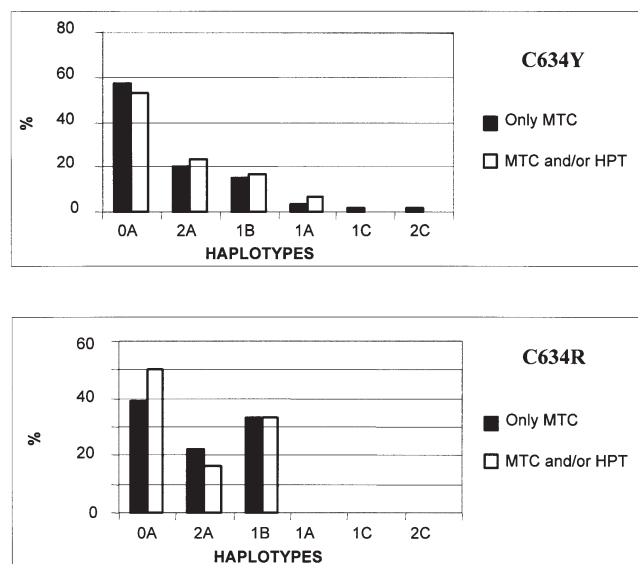
Table IV. *RET* haplotypes associated to each kind of oncogenic mutation.

Mutation	Haplotype	No. of families	Family phenotype[a]
Cys634Tyr	0A	7	MEN 2A
	2A	3	MEN 2A
	1B	1	MEN 2A
	?	1	MEN 2A
Cys634Arg	1B	2	1 MEN 2A and 1 FMTC
	0A	2	1 MEN 2A and 1 PTC
	2A	1	FMTC

[a]The family phenotype refers to the initial diagnosis based on clinical/biochemical results and study of familial history, previous to the molecular analysis. ?, in this family the *RET* haplotype could not be deduced because the data were not informative.
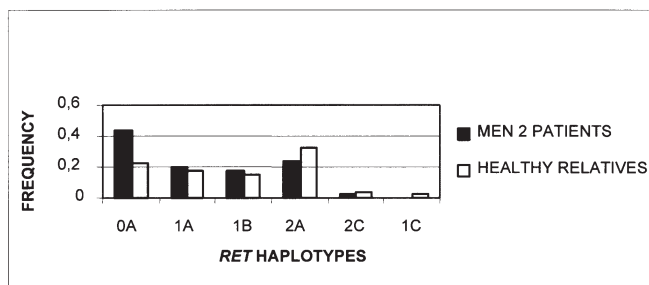
Figure 2. Distribution of *RET* haplotypes in MEN 2 patients and their healthy relatives.

Figure 3. Haplotypic distribution in patients carrying the same *RET* mutation with a different clinical presentation.

haplotypic distribution was similar among our MEN 2 patients and their healthy relatives in the context of each family, and also in a global sense (Fig. 2). The values of frequency were very similar to those obtained in the general Spanish population (28,29,31,32).

In addition, we proceeded to analyze the distribution of the *RET* variants and haplotypes for those 4 FMTC families with no oncogenic mutation identified, in order to evaluate their association with the manifestation of the disease. This analysis led us to observe that the 4 FMTC families were linked to *RET*, since in each pedigree the affected members shared the same specific haplotype. However, when comparing among the 4 families, we found variability regarding the specific haplotype which seemed to segregate with the disease (2 families with haplotype 1A, 2 with haplotype 1B, and 2 with haplotype 0A).

By performing the genotype-phenotype analysis we confirmed the association of the mutations affecting codon 634 and the presence of PTC or PTC + HPT in the families (Fisher's exact test with two tails, p=0.03). Nevertheless, there existed inter- and intra-familial phenotypic variability among the patients carrying one of those specific *RET* mutations (Table IV; C634Y or C634R). Thus we proceeded to analyze and compare the distribution of the poly-morphisms/haplotypes in those groups of patients, in order to evaluate them as modifier elements. However, a random transmission, either *cis* or *trans*, of the 8 variants tested was found, so that no association was observed between any polymorphism and the presence of PC and/or HPT. In addition no specific haplotype was found to correlate with the presentation of the disease (Fig. 3).

Discussion

In this report we present a comprehensive study of molecular genetic screening of Spanish families with any sign typical of MEN 2 syndromes. Some parts of the families included

common ancestors. However, as shown in Table IV, we observed that the most frequent mutations present in our families, C634Y and C634R, were associated with several different haplotypes. We also found that the allelic and

here have been partially presented in previous reports (10,23,24,35,36). Germline mutations were found in 22.8% of the probands of our MTC families, and in 1 patient presenting with PC. Our results are in concordance with the data stating that up to 25% of MTC cases are inherited (2,5,6). In FMTC families our detection rate was only 50% (4 out of 8 families), probably due to the application of milder clinical criteria for this group. For our 4 FMTC unsolved families, the disease could still be a part of an inherited syndrome where the mutation may reside in other exons of *RET*, just as it occurs with some FMTC pedigrees previously published (37,38). In this sense, our haplotype analysis confirms that all these families are indeed linked to *RET*. However, the inheritance of some mutations lying in other genes cannot be completely excluded.

Again our data seem to confirm the association of mutations affecting codon 634 and the presence of phaeochromocytoma or parathyroid disease observed in the International *RET* Mutation Consortium series (5). As previously postulated (39), this apparent association might be due not to a direct effect of the resultant amino acid substitution on susceptibility to PC or HPT disease, but to the effect of a founder chromosome which carries both the codon 634 mutation, and another tightly linked variant determining the probability of PC/HPT involvement. However, our results derived from the haplotype analysis revealed that each of the 634 mutations is represented by more than one haplotype, thus suggesting that the oncogenic mutations themselves are responsible for the final phenotype in the families.

On the other hand, with this haplotype analysis we also sought to investigate the phenotypic inter- and intra-familial variability among patients carrying the same mutation at codon 634. Because PTH and/or PC were not a consistent feature in all the patients who carried mutations affecting codon 634, there must exist other factors, which might modify the expression of the mutation with respect to PC/PTH manifestations. It is supposed that if the presence of a polymorphism correlates (or associates) with a change in phenotype, it would be plausible to infer that such polymorphism indeed acts as a modifier. Thus, we proceeded to analyze a selection of *RET* variants as putative modifier loci. Among those polymorphisms we had selected G691S and S904S because of their previous definition as genetic modifiers of MEN 2A by Robledo *et al* (19). They found a strong correlation between both variants and the age of onset of MEN 2A. It was then proposed that such specific effect of G691S probably could be attributable to some cooperative action on RET-dimerization with the oncogenic mutations. Alternatively, it was also postulated that G691S might create a new site susceptible to serine-phosphorylation affecting to some extent the downstream signaling events. In addition, given that S904S does not lead to an amino acid substitution, the most plausible explanation for the previous results, would be the complete linkage with G691S. Nevertheless, it has been also proposed that S904S might influence *RET* expression by affecting RNA stability (19). Unfortunately, given that the clinical diagnosis for some members of the same family are conditioned on the time of genetic diagnosis of the corresponding proband, and that some members may have developed the disease immediately before the study was

performed, the age at the onset of the disease is only truly known for the index cases. Because of the small sample size of our MEN 2A probands, the correlation between the age of onset and the polymorphisms/haplotypes could not be reliably performed. However, we could examine the effect of both variants in the appearance of PC/HPT, but no association was found, in agreement with previous reports (19).

In addition, we have analyzed S836S and the three intronic IVS1-1463T→C, IVS1-1370C→T and IVS1-126G→T, because of the association of such variants themselves or of specific haplotypes comprising them, to the sporadic forms of MTC or PTC (28,33,34,40,41), which makes them good candidate variants to act as a modifier loci for MEN 2. Moreover, we have also selected two SNPs at -5 and -1 from the transcriptional start site (-200A→G and -196C→A), given that results derived from luciferase expression assays have shown differential activities for their different combinations (AC, GC and GA), suggesting a role of those variants in the transcriptional regulation of *RET* (29,42,43). Thus, we postulated that the two polymorphisms may also exert a modifier effect on MEN 2 phenotypes. Nevertheless, none of the *RET* variants here tested, nor any specific combination of them were found to be associated with the presence of PC and/or HPT. Our results, however do not exclude the possible role of other *RET* variants in the final presentation of the disease. Further studies are warranted in order to elucidate if other polymorphic variants located both in *RET* and other candidate genes may act as modifier elements of the disease.

Acknowledgements

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