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Polymorphisms G691S/S904S of *RET* as Genetic Modifiers of MEN 2A[1]

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ABSTRACT

Multiple endocrine neoplasia type 2A (MEN 2A) is associated with specific germ-line missense mutations in the *RET* proto-oncogene. Only a minor fraction of human disorders are simple monogenic diseases, and the identification of polymorphisms that increase susceptibility, including variations in pathological phenotypes, to human diseases is one of the key problems in medical genetics. To explore this idea, we analyzed the polymorphisms G691S (exon 11) and S904S (TCC-TCG, exon 15) of *RET* in 198 individuals corresponding to 35 unrelated Spanish MEN 2A families (104 patients with oncogenic MEN 2A mutation and 94 healthy relatives). We found strong cosegregation between both polymorphisms (100% Fisher's exact test, $P < 0.001$) using a control population containing 653 healthy individuals (362 females and 291 males). Interestingly, we found that the homozygous for these polymorphisms were, on average, 10 years younger at diagnosis compared with heterozygous and wild-type homozygous ($P = 0.037$). Taken together, all these findings could indicate that the G691S and S904S variants of *RET* have a modifier effect on the age at onset of MEN 2A. Moreover, compared with the control population, the homozygote status was significantly more prevalent in a series of 110 sporadic thyroid carcinoma (odds ratio = 2.36), suggesting that these polymorphisms may play a role as a low penetrance risk factor.

INTRODUCTION

MEN 2[4] shows three clinically distinct forms: (*a*) MEN 2A; (*b*) MEN 2B; and (*c*) FMTC. All forms are transmitted as an autosomal dominant trait with a high degree of penetrance and variable clinical expression. The presence of MTC is a common clinical feature. In patients with FMTC, only the thyroid gland is affected, whereas patients with MEN 2A may develop phaeochromocytoma and primary hyperparathyroidism. Furthermore, MEN 2B (OMIM 162300) are characterized by mucosal neuromatosis, ganglioneuromas of the intestinal tract, and a marfanoid habitus (high-arched palate, pectus excavatum, bilateral pes cavus, high patella, and scoliosis).

It has been shown that specific germ-line point mutations of the *RET* gene are responsible for the three forms of MEN 2 (1, 2). The *RET* proto-oncogene encodes a tyrosine kinase receptor implicated in neural crest tissue development and differentiation. The GDNF has been recognized as a ligand for RET, and RET activation requires the formation of a multimeric receptor complex that includes GDNF as ligand and a glycosylphosphatidyl inositol-anchored protein termed GDNF receptor-α that functions as coreceptor. Independently of GNDF and GDNF receptor-α, the multiprotein RET signaling complex can contain other ligands and coreceptors.

The majority of MEN 2A cases have germ-line missense *RET* mutations involving one of six highly conserved cysteines (codons 609, 611, 618, and 620 in exon 10 and codons 630 and 634 in exon 11) of the extracellular cysteine-rich domain (1, 3, 4). In patients with FMTC, *RET* mutations are mainly detected in the same six codons as for MEN 2A and also in codon 768 (exon 13; Ref. 3), or in codon 891 (exon 15), which lies in the intracellular region of *RET* (5). Mutations of codon 918 in *RET* exon 16, which lies within the RET tyrosine kinase domain, have been identified in the majority of patients with MEN 2B (2), and this mutation appears to alter the substrate specificity of the RET tyrosine kinase (6). Furthermore, several studies have identified the presence of *RET* mutations in sporadic MTC (codons 918 and 768) and phaeochromocytoma (codon 918) tumor tissues but not in constitutional DNA from the same patient (4).

A low penetrance gene is defined as a gene in which subtle sequence variants or polymorphisms may be associated with a small to moderate increased relative risk for the development of the disease. Such variants are relatively common in the population and, as such, may confer a much higher attributable risk in the general population than rare mutations in high penetrance cancer susceptibility genes. On the other hand, if the presence of a polymorphism correlates (or associates) with a change in phenotype (clinical characteristics of the disease), it would be possible to infer that such polymorphism indeed acts as a modifier. Taking a single type of MEN 2, there are variations between members of the same family regarding the clinical presentation of the disease and the age at onset (7). Variations in phenotypes within the same family suggest a role for genetic modifiers, which may also work through quantitative effect (7). In a previous study on four Spanish families with MEN 2A, we detected two polymorphisms of *RET* in strong linkage disequilibrium, G691S (exon 11) and S904S (TCC-TCG, exon 15; Ref. 8). Here, we analyzed 35 Spanish families with MEN 2A to explore whether these two RET polymorphisms, G691S and S904S, could have any influence on the clinical form and age at onset of the disease. Moreover, the influence of these polymorphisms on 110 cases of sporadic MTC was also explored.

MATERIALS AND METHODS

Patients and DNA Preparation. We ascertained 35 independent Spanish families as having individuals affected by MEN 2A. Venous blood was obtained, after informed consent was given, from 200 family members, 104 containing *RET* oncogenic mutation and 96 unaffected, for DNA studies. Similarly, we studied 110 patients with MTC, considered as sporadic tumor because germ-line mutation had not been detected in exons 10, 11, or 13–16, and the patient had no familial history of the disease. Diagnosis of MTC, primary hyperparathyroidism, and phaeochromocytoma was based on documented pathological examination. DNA was extracted from blood lymphocytes according to standard procedure (8). Likewise, genomic DNA was obtained from 653 unrelated healthy individuals (362 females and 291 males). They were anonymous samples roughly frequency matched by age and sex with the clinical cases.

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[4] The abbreviations used are: MEN 2, multiple endocrine neoplasia type 2; MTC, medullary thyroid carcinoma; FMTC, familial medullary thyroid carcinoma; OR, odds ratio; GDNF, glial cell line-derived neutropic factor.

Amplification and Sequencing Analysis. Genomic DNA was amplified by PCR using primers described previously (3, 5, 8) and the GeneAmp PCR System 9700 thermocycler (Perkin-Elmer Corp., Norwalk, CT). The reaction was carried out with 0.5–1 unit of Taq polymerase (Roche, Indianapolis, IN), 5 μl of 10 $\times$ PCR amplification buffer, 0.8 μM each primer, 0.2 mM each deoxynucleoside triphosphate, and double-distilled water to a final volume of 50 μl. DNA was denatured previously at 94°C for 5 min, and then 30 PCR cycles were run under the following conditions: DNA denaturation at 94°C for 30 s, primer annealing at 58°C for 25 s, and DNA extension at 72°C for 30 s, with a final extension of 5 min at 72°C. The PCR products were tested by agarose gel electrophoresis (3% agarose gels in Tris-borate-EDTA buffer) and purified with E.Z.N.A. Cycle-Pure Kit (Omega Bio-tek) according to the manufacturer's instructions. The purified products were subsequently sequenced using an ABI Prism 3700 automatic sequencer (Applied Biosystems, Perkin-Elmer). The reaction was carried out in 4 μl of Big Dye terminator cycle sequencing Kit (Perkin-Elmer), 10 pm of the sense/antisense primer, 5% DMSO, and 6–12 ng of amplified DNA.

G691S/S904S Analysis by Pyrosequencing System/Restriction Enzyme. The genotyping of the G691S polymorphism was performed using the Pyrosequencing technology. The design of PCR and sequencing primers was performed according to the guidelines supplied by Pyrosequencing AB. The biotinylated PCR products (25 μl) were immobilized onto streptavidin-coated Dynabeads (Dynal Biotech ASA) and prepared for analysis using the standard protocols from Pyrosequencing. The polymorphic positions were analyzed using a PSQ 96 System, SNP software, and SNP Reagent kits (Pyrosequencing AB). The variant S904S was analyzed by Rsa I restriction enzyme.

Statistical Methods. Hardy-Weinberg equilibrium for G691S/S904S polymorphisms was assessed in a sample of 653 controls using the likelihood ratio test. The prevalence of G691S/S904S polymorphisms was compared in MEN 2A cases and their healthy relatives using the corrected Pearson's χ^2 test, allowing for the correlation between members of the same family (9). In the same way, a possible correlation among cases between these polymorphism and either the clinical presentation or the mutated codon in *RET* was assessed. The relationship between G691S/S904S polymorphism and age at diagnosis in MEN 2A patients was investigated in a double way, considering "age" as a continuous variable and also dichotomous one, taking 20 years as the cutoff. Differences across G691S/S904S groups were quantified using linear regression and logistic regression. In both instances, Huber & White robust estimators of variance were used, considering that patients are clustered in families as a way to take the correlation between the members of the same family into account (10). The same analysis was restricted to index cases or probands, which represent a subgroup of patients for which their age at the onset of clinical symptoms was truly known. This group served to test the apparent association between G691S/S904S polymorphism and an earlier start of the disease. Finally, the possible association between G691S/S904S polymorphisms and sporadic MTC was explored, comparing the group of controls with 110 sporadic cases using logistic regression. This relationship was also separately studied in two age groups using a cutoff of 45 years, given that sporadic cases tend to appear at an older age. A possible interaction between age and G691S/S904S polymorphism was tested introducing the corresponding factor in the model. All of the statistical analyses were performed using STATA (10).

RESULTS AND DISCUSSION

Previously, we found two variants of *RET* gene, G691S (exon 11) and S904S (TCC-TCG, exon 15), that cosegregated together as haplotype (100% Fisher's exact test, $P < 0.001$), suggesting that these polymorphisms are in linkage disequilibrium with each other (8). The 100% cosegregation was corroborated again in the present study. Interestingly, this *RET* haplotype displayed a different distribution ($P = 0.007$) according to the age at onset of MEN 2A patients (8). In the present study, we screened the G691S/S904S haplotype in 35 unrelated Spanish MEN 2A families with a total of 200 members studied, and 104 of them were MEN 2A patients containing *RET* oncogenic mutations. The presence of the polymorphisms was independent of the specific pathogenic mutation of *RET* in MEN 2A families (data not shown). Furthermore, we did not observe any relationship between these polymorphisms and the type of clinical presentation (MTC, phaeochromocytoma, and primary hyperparathyroidism; data not shown). Homozygous cases were, on average, 10 years younger when they were diagnosed (Table 1 and Fig. 1; $P = 0.037$). We decided to categorize the age at onset of symptoms into two levels, taking the rounded 33rd percentile of the observed distribution as cutoff. Homozygous had an 8-fold probability to be diagnosed at an age before 20 compared with wild-type cases (Table 1; $P = 0.01$). Restricting the analysis to cases with RET mutation in codon 634 (the most frequent altered codon that included all homozygous in our study) still yielded a similar result, with a statistically significant OR > 6 (Table 1). Obviously, these results could be biased given that the clinical diagnosis for some members of the same family may be conditioned on the time of genetic diagnosis of the corresponding proband. For this reason, we decided to repeat the analyses taking only the index case for each family. When we carried out the genetic analysis focusing specifically on probands (something logical for a genetic syndrome), which were diagnosed when they showed clinical symptoms, a statistically significant ($P < 0.001$) relation between the presence of homozygote G691S/S904S haplotype and age at onset of symptoms was observed (Table 1). The relative risk for earliest clinical symptoms was substantially higher if we considered only the MEN 2A probands (Table 1). In this case, the OR for the

Table 1 *Relationship between G691S/S904S polymorphisms and age at onset of symptoms for MEN 2A patients*

	Without tumor		With tumor	Age at onset of symptoms			Mean		Diagnosed at age <20[b]			
G691S/S904S	<20 y	≥20 y	patients	Mean	P50	P25–P75	difference[a]	$P^{a,c}$	*n*	%	OR[b,d]	$P^{b,c}$
All cases												
Wild type	7	14	45	30	28	22–40			8	14%	1.00	
Heterozygous	3	6	22	31	31	18–41			6	21%	1.77	0.440
Homozygous	0	0	7	20	17	11–26	−10.33	0.037	4	57%	8.50	0.010
Cases with RET mutation in codon 634												
Wild type	3	6	34	28	27	22–33			7	18%	1.00	
Heterozygous	2	3	18	29	28	18–39			6	29%	1.89	0.425
Homozygous	0	0	7	20	17	11–26	−7.93	0.104	4	57%	6.29	0.030
Probands				Mean	P50	Min–max						
Wild type			20	35	34	15–53			2	10%	1.00	
Heterozygous			12	35	36	17–50			1	8%	1.00	
Homozygous			3	15	11	11–24	−19.42	<0.001	2	67%	19.3	0.033
Probands mutation codon 634				Mean	P50	Min–max						
Wild type			14	33	32	15–53			2	14%	1.00	
Heterozygous			9	35	35	17–50			1	11%	1.00	
Homozygous			3	15	11	11–24	−18.71	<0.001	2	67%	13.3	0.064

[a] Excluding people without tumor comparing ++ *versus* −− and −/+.
[b] Excluding people without tumor younger than 20.
[c] *P* using the Huber and White robust estimation of variance considering patients clustered in families.
[d] Odds Ratio: relative risk of having an early start taking G691S/S904S as reference.

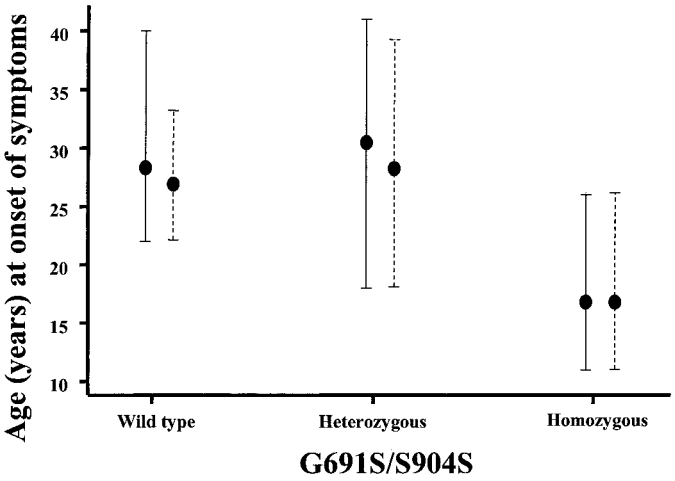

Fig. 1. Age at onset of symptoms of MEN 2A patients *versus* G691S/S904S polymorphisms. This graphic represents the age at symptom onset of MEN 2A patients (median and P25–P75) *versus* its genetic background of G691S/S904S polymorphisms. *Solid lines*, all MEN 2A patients analyzed; *broken lines*, only the MEN 2A patients containing the *RET* codon 634 oncogenic mutation. *Black points*, the median values.

homozygous G691S/S904S was 19.3 (13.3 when we analyzed only probands containing the *RET* codon 634 oncogenic mutation). Taken together, these results suggest that the presence of the polymorphisms G691S/S904S of *RET* could be related with early appearance of symptoms in MEN 2A patients, and these polymorphisms could be considered as genetic modifiers. The polymorphisms G691S and S904S of *RET* were described previously (11), and until now, it is not known whether these germ-line variants play other interacting, predisposing, or modifying roles in the pathogenesis of MEN 2A. We analyzed the allele frequency of G691S/S904S in a set of 653 controls (unrelated healthy individuals: 362 females and 291 males) and observed that the genotype distribution of this haplotype did not deviate significantly from the Hardy-Weinberg equilibrium, independently to test either the total sample or separately by sex and groups of age (data not shown).

The precise mechanism by which these polymorphisms may affect the age of onset is unknown and open to speculation. The variant G691S (exon 11) occurs in the cytoplasmic tail of RET, between the transmembrane region and first tyrosine kinase domain and close to the residue Y687 that was found phosphorylated in all MEN 2A cases, but only in some MEN 2B patients (11). Although G691S is not considered as oncogenic mutation, we cannot exclude a functional role based on quantitative effects. In this regard, we carried out the genetic analysis in a group of 110 cases of sporadic MTC and did not detect any relationship between G691S/S904S polymorphisms and the age at onset of symptoms (data not shown). However, compared with healthy controls, the prevalence of G691S/S904S homozygous state was higher among sporadic MTC patients (Table 2; OR = 2.36, $P = 0.045$), suggesting that these variants may play a role as a low penetrance gene. Even though we did not find a statistically significant interaction between the effect of these variants and age, the risk excess observed for homozygous was more noticeable among younger cases (Table 2; OR = 3.42, $P = 0.036$), for whom the influence of genetic risk factors must be stronger.

RET mutations of sporadic MTC and MEN 2B lie within the RET tyrosine kinase domain (2, 4) in sharp contrast with *RET* mutations of MEN 2A, which lie in the RET extracellular cysteine-rich domain (1, 3, 4). Taken together, all these data suggest that the specific effect of G691S polymorphism on MEN 2A pathologies probably could be attributable to some cooperative action on RET-dimerization with the oncogenic mutations of the extracellular RET cysteine-rich domain. This hypothetical mechanism could not have any effect on sporadic MTC and MEN 2B because their *RET* oncogenic mutations (on the tyrosine kinase domain) appear to alter the substrate specificity of the RET tyrosine kinase, and their transforming activities, in contrast with MEN 2A, are independent of RET dimerization (6). A second hypothesis to explain the modifier effect is that the change glycine to serine in codon 691 originates a new site susceptible to serine-phosphorylation and thereby might activate, or have some effect, on the downstream signaling events. A similar effect has been described recently affecting the α_{2A}–andrenergic receptor in which the authors hypothesized that residues of this type may act as a physiologically relevant switch (12). Furthermore, a similar mechanism has been proposed for the phosphorylation of serine 696 in RET (13).

Because S904S (TCC-TCG, exon 15) does not lead to an amino acid alteration, it is difficult to imagine how this conserved polymorphism may affect the RET activity. A plausible explanation is that, because of the 100% cosegregation, the results obtained with S904S could be interpreted as a founder effect without influence as genetic modifier, according to a role of the silent sequence variant (S904S) as a linked neutral polymorphism, and the main putative modifier would be the amino acid sequence variant G691S. However, it is possible that the sequence variant S904S also influences *RET* expression, such as influencing RNA stability. In this regard, it has been shown that polymorphic sequence variants (SNPs) can lead to production of different amounts of mRNA (14), cause different structural folds of mRNA (15), and originate alterations of splicing (16). Specifically, the rare *RET* sequence variant S836S (AGC-AGT) may play a role in

Table 2 *Relationship between G691S/S904S polymorphisms and sporadic MTC cases compared with healthy controls*

	Healthy controls		Sporadic MTC cases					
Groups	*n*	%	*n*	%	P^a	OR[d]	95% Confidence interval[b]	P^b
All ages					0.111			
Wild type	422	65%	71	65%		1.00[c]		
Heterozygous	210	32%	31	28%		1.00[c]		
Homozygous	21	3%	8	7%		2.36	1.01–5.47	0.045
Younger than 45 years					0.098			
Wild type	332	65%	24	60%		1.00[c]		
Heterozygous	160	32%	12	30%		1.00[c]		
Homozygous	16	3%	4	10%		3.42	1.08–10.8	0.036
45 years and older					0.471			
Wild type	90	62%	47	67%		1.00[c]		
Heterozygous	50	34%	19	27%		1.00[c]		
Homozygous	5	3%	4	6%		1.70	0.44–6.55	0.443

[a] Fisher's exact test.
[b] Confidence intervals and *P* using the Huber and White robust estimation of variance.
[c] Both categories combined as reference.
[d] Odds Ratio: relative risk of having an early start taking G691S/S904S as reference.

the genesis of sporadic MTC (17), and it has been noticed that two polymorphisms of *RET*, A45A (GCG-GCA; exon 2) and L769L (CTT-CTG; exon 13), show a strong association with Hirschsprung disease (18, 19).

In light of these results, our hypothesis is that the G691S/S904S haplotype of *RET* may somehow influence the age at onset in MEN 2A patients and could be considered as a genetic modifier of this pathology. Moreover, our data indicate that they could also play a role as a low penetrance gene in sporadic MTC cases. Interestingly, these two polymorphisms appear to be underrepresented in Hirschsprung patients compared with controls, suggesting that might protect against the development of that disease (19). Germ-line, loss-of-function mutations of the *RET* proto-oncogene are associated with a small subset of Hirschsprung disease or aganglionic megacolon, which is a congenital disorder characterized by the absence of enteric ganglia along a variable length of the intestine (OMIM 142623). If the pathogenic/modifier effect cannot be associated with the G691S/S904S variants of *RET*, then the possibility has to be considered that the base substitution is in linkage disequilibrium with an unknown functional variant upstream or downstream. Finally, it is conceivable that genetic modifiers of the primary oncogenic mutation could operate through effects on the timing of RET expression (7). This idea has been suggested in the context of three founder lines of mos transgenic mice that develop different patterns of MEN 2-related tumors on different genetic backgrounds (20). At present, our understanding of the interplay at the cellular level between inherited cancer modifier genes with oncogenes and tumor suppressor genes is rudimentary. The associations highlighted here might deserve to consider G691S/S904S polymorphisms as molecular epidemiology markers in MEN 2A and sporadic MTC, and it remains for further biochemical and functional studies to elicit the mechanism by which these variants indeed influence the RET activity.

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